Effect of stress and diapause in two Calliphoridae species.

by

Bobbie Johnson

A thesis submitted to The University of Birmingham
for the degree of DOCTOR OF PHILOSOPHY

School of Biosciences
The University of Birmingham
January 2013

Abstract

Cultures of two Dipteran flies (*Calliphora vicina (*R-D) and *C. vomitoria* (L.)) were established to answer questions in regards to responses to thermal and desiccation stress, effects of diapause and the mechanisms which underpin diapause. The findings are divided in to two sections.

Unequivocal new findings – *Calliphora vomitoria* were seen to depend on water being present in culture medium for survival to be high. Furthermore, *C. vomitoria* were found to be less able to resist desiccation than *C. vicina.* Larvae of *C. vicina* and *C. vomitoria* showed different cold tolerance strategies, with *C. vicina* being freeze-avoiding and *C. vomitoria* 'partially' freeze-tolerant. Metabolomics, using ^{1}H-NMR, revealed that diapause and non-diapause had distinct metabolic profiles. Diapause larvae were seen to reduce energy synthesis from the Krebs cycle and increase glycolysis. *Calliphora vicina* and *C. vomitoria* also exhibited different diapause phenotypes; *C. vicina* entered a maternally regulated facultative diapause as an L3 larvae, *Calliphora vomitoria* had a less distinct diapause, with maternal conditions having little effect.

Speculative new findings - Despite the above differences in culturing, desiccation and cold tolerance, *C. vicina* and *C. vomitoria* were able to produce a viable cross, though field fresh *C. vomitoria* were not used, as such it cannot be confirmed if this could occur in the wild. Field studies showed that increased temperatures due to climate change may affect both phenology and survival of insects; *C. vicina* was seen to have a delayed induction to diapause and a reduction in the proportion entering diapause. Diapause conferred increased cold tolerance; as such those insects that overwinter not in diapause may suffer increased mortality. This study only examined one aspect of climate change – increased temperatures. To get a more holistic view an experimental design including precipitation, U.V. and thermal extremes is needed.

This research shows how even closely related species can evolve different mechanisms to cope with stress, it is hoped that through RNAi and or epigenetics that this research will be furthered.

Acknowledgements

I would like to thank NERC for sponsoring my PhD, and to my supervisors Scott Hayward and Jeff Bale for writing the project proposal and supporting me through the whole process.

I was privileged to be part of a wonderful lab group at Birmingham University. Lucy and Steve offered much needed help while I was setting up the lab, and later on providing help with statistical analysis. My greatest gratitude goes to Gwen who not only provided invaluable insight in to lab life but became one of the greatest friends anyone could ask for. Thanks also go to my 'lab partner' Paul Coleman who kept me entertained throughout the many hours of counting and sorting smelly maggots and to Emily who was a late entrant in to 'the dream team'. My thanks also go to Megan, Ji and Matt who not only proof-read parts of my thesis but kept me fit during the many hours of chasing a small ball around the squash courts. I really will miss our lab lunches and outings, and I thank each of you for creating a wonderful atmosphere in which to spend three and a half years.

Special thanks go to John for simply being there when I needed him most and helping even with the most mundane and sole-destroying of tasks. I really can't thank you enough!

My final thanks go to my family; my brother Martyn, who endeavors to keep both my feet firmly on the floor, and especially, to my parents for convincing me to do a degree in the first place. I have loved each of my years at university; and I am truly thankful for everything my parents have done for me.

Table of Contents

List of Figures

Figure 1.9. Global temperature trend from 1958 to 2008 (data and mapping algorithm from NASA Goddard Institute for Space Studies). Figure taken from MacDonald, (2010).

Figure 1.10. Life cycle of Calliphora vicina. Adults lay eggs upon meat, which hatch and progress through 1st (L1), 2nd (L2) and 3rd (L3) instar stages. During the L3 stage, larvae stop actively feeding and move away from the meat (termed wandering). During this stage, larvae will either remain as larvae and enter diapause, or pupate and continue development to adults. Diapause incidence is determined by adults (sensitive stage) experiencing the critical day length (CDL), together with appropriate temperature cues.

Figure 1.11. Temporal abundance of *Calliphora vicina* and *Calliphora vomitoria* from June 2001 to October 2002. Vertical axis shows the number caught per day. Taken from Hwang & Turner (2005).

Figure 2.1. Schematic of fly trap used to catch *C. vicina* at the University of Birmingham (52^{o}N). Collection chamber is made of two plastic bottles inserted inside each other. The bait chamber is constructed from the lower portion of a plastic bottle, and holds a vial of sodium hydroxide containing a small amount of ox blood, and a weigh boat containing 15g of ox liver. Figure modified from Hwang and Turner (2005).

Figure 2.2. Photo of *Calliphora vicina*. Key distinguishing features include the orange jowls, black bristles on the chin and orange basicosta. Photo taken from Natural History Museum, (2011).

Figure 2.3. Photo of *Calliphora vomitoria*. Key distinguishing features include the black jowls, orange bristles on the chin and black basicosta. Photo taken from Wageninger, (2011).

Figure 2.4. Mean number of days (±SE) for larvae to cease active feeding (wander) after oviposition, when held at 11^{o}C. Adults of *C. vicina* and *C. vomitoria* were placed in a cage held at 20^{o}C and exposed to either a long day (LD) or a short day (SD) photoperiod. Larvae were monitored daily and the day of mass wandering (exit off liver) was noted. A total of 19 mass wandering events were recorded for LD *C. vicina*, N=18 SD *C. vicina*, N=14 LD *C. vomitoria* and N=18 SD *C. vomitoria*. Groups that are not significantly different are marked with an asterisk.

Figure 2.5. Mean percentage (±SE) of individuals remaining as larvae 30d post oviopsition in *C. vicina* and *C. vomitoria*. Two photoperiods were established for adult cultures, short day (SD) and long day (LD). Larval progeny were held at 11^{0}C until day 30 post oviposition, when the proportion remaining as larvae was calculated. Groups that are not significantly different are marked with an asterisk.

Figure 2.6. Mean percentage (±SE) remaining as larvae at 30, 40, 50 and 60d post oviposition for *C. vicina* (diamond) and *C. vomitoria* (square). Larvae were held at 11^{o}C (darker lines, n = 80) or at 5^{o}C (lighter lines, n=30) in constant darkness. Larvae used were progeny of adults held at an SD photoperiod.

Figure 2.7. The effect of moisture level on the number of days to oviopsition in *C. vomitoria*. Larvae were progeny of short day adults (held at 12:12 photoperiod) and cultured at 20^{o}C; subsequent larvae were held at 11^{o}C until 2-3 days before wandering and then held at 5^{o}C. On the day of mass wandering, larvae (n=20) were either placed in media with a

water:sawdust mass ration of 0:1, 1:1 or 2:1 at 5°C. The numbers of pupae and larvae were recorded daily.

Figure 2.8. Mean percentage of population that were female for *C. vicina*, *C. vomitoria*, F1 and F2 P ♀ *C. vomitoria* (n=40 for all samples). As the frequency of females was recorded from lone populations standard error bars could not be produced.

Figure 3.1. Example output of the exotherms of eight L3 non-diapause (ND) larvae of C. vicina. Readings were obtained using K-type thermocouples attached to a picotech TC-08 datalogger. Larvae were placed in Beem capsules, in contact with the thermocouple, and were cooled from 11°C to -33°C at 0.5°C min-1.

Figure 3.2. Mean SCP (±SE) of different developmental stages in the life cycle of a) *C. vicina* and b) *C. vomitoria*. Adults were reared under either long day (LD) (light grey) or short day (SD) conditions (dark grey), and the SCP of their progeny through to adulthood was assessed (n=16). Significant differnces between LD and SD are indicated with an asterisk.

Figure 3.3. The mean SCP (±SE) of *C. vicina* and *C. vomitoria* when either inoculated with ice (termed 'Ice inoc') or held in dry conditions (termed Dry SCP). The SCPs of dry samples were determined by cooling at a rate of 0.5°C min^{-1}, the SCPs of ice inoculated samples were determined by cooling at 0.2°C min^{-1}. Groups that share the same letter are significantly different to each other.

Figure 3.4. Survival to pupariation (light grey) and eclosion (dark grey) of *C. vicina* (n=16), *C. vomitoria* (n=22) and their cross (n=17) when inoculated with ice at c. -1.5°C during cooling from 5°C to -33°C at 0.2°C min^{-1}; after their SCP was reached, larvae were removed. The percentage pupariation and survival are represented as mean values (±SE). Groups that share the same letter are significantly different to each other

Figure 3.5. Survival to pupariation (light grey) and eclosion (dark grey) of *C. vicina* (n=24) and *C. vomitoria* (n=70), when inoculated with ice at c. -5.5°C during cooling from 5°C to -5.5°C at a rate of at 0.2°C min^{-1}. Larvae were then held at -5.5°C for 1h before being re-warmed at 0.2°C min^{-1}. The percentage surviving to pupariation and eclosion are represented as mean values (±SE). Groups that share the same letter are significantly different to each other

Figure 3.6. Mean percentage (±SE) of larvae surviving to eclosion after 2h at -13°C (the discriminating treatment). Four pre-treatments were established, a 2h acclimation at either 0°C, -2°C, -5°C or a ramp from 11°C to -13°C at 0.5°C min^{-1}, before a 2h exposure to the discriminating temperature. Groups that share the same letter are significantly different to each other

Figure 4.1. Airtight desiccation chamber, containing an open microcentrifuge tubes or glass vials which are surrounded to the shoulders by Drierite™ and sealed within an airtight container.

Figure 4.2. Mean percentage water loss of individual L3 larvae of *C. vicina, C .vomitoria* and their cross, following different durations of exposure to <5% RH at 5°C. N=8 for all experiments.

Figure 4.3. Mean percentage water loss of L3 larvae of *C. vicina* (a) and *C. vomitoria* (b) following different durations of exposure to low humidity. Larvae were either held in groups and

placed in glass vials (N=8), or were individually held in 5ml microcentrifuge tubes. All larvae were surrounded by Drierite™ at 5°C/ <5%RH.

Figure 4.4. Mean survival to eclosion of individual L3 larvae of *C. vicina* (termed 'C. vicina'), *C. vomitoria* (termed 'C. vomitoria'), their cross (termed 'cross') and groups of *C. vomitoria* (termed 'C. vomitoria group') following different periods spent at <5% RH at 5°C. In the individual experiments, eight individual larvae of each of the three species were removed daily for 14 days. For group experiments, three vials each containing ten larvae of *C. vomitoria* were removed daily for 7 days. Once larvae were removed they were transferred to 20°C; *C. vicina* larvae were held in dry sawdust until eclosion occurred, *C. vomitoria* and the cross were placed in damp sawdust until eclosion occurred. Standard error is not included as only one trial was conducted using eight individual larvae.

Figure 4.5. Mean percentage (±SE) of L3 *C. vomitoria* larvae pupating and eclosing after exposure to control and cross tolerance treatments. For control samples, glass vials containing eight larvae were held at 5°C for 24h and then exposed to -13°C for 2h (N=8), larvae were then held at 20°C until larvae eclosed. Cross tolerance (cross tol) treatment involved holding vials containing eight larvae in Drierite™ at 5°C for 24h prior to being exposing to -13°C for 2h (N=8). Groups with the same letter are significantly different to each other.

Figure 5.1. Schematic depiction of temporal development of *Calliphora vicina*, including development (green block), diapause (red block) and post-diapause quiescence (yellow block; termed quiescence). Dark sold arrow represents the current critical day length (CDL), which occurs in early Autumn. The dotted arrows represent the timing of the transition between the different stages, each of which is thought to vary depending on temperature (red arrows). Figure modified from Hayward *et al*., unpublished.

Figure 5.2. Schematic of the construction of containers used to house larvae used in all three field sites (open, woodland and cloche).

Figure 5.3. a) open site; b) woodland site; c) cloche site, d) aerial photo shows the location of the three sites in relation to each other. All located on the University of Birmingham campus, U.K.

Figure 5.4. The mean percentage (±SE) of larvae in diapause at 30 days post-oviposition. Cages were established on 28th August, 14th September and 9th October in 2009 (grey bars) and 2010 (black bars). ^ designates trials where the percentage of larvae in diapause could not be recorded due to larvae being at the second instar stage, not the third instar in which diapause occurs.

Figure 5.5. Number of days for adults to oviposit in field cages set up on 28th August (termed 'Aug'), 14th September (termed 'Sept') and 9th October (termed 'Oct') trial in 2009 (grey) and 2010 (black) (N=1 cage per trial).

Figure 5.6. **August 2009 trial**. The maximum (red square), minimum (green triangle) and mean (blue diamond) soil temperatures at 5cm sampled every 3 minutes at the open site, wood site and cloche, assessed from 14th September 2009 to 14th October 2009

Figure 5.7: September 2009 trial a) The maximum (red square), minimum (green triangle) and mean (blue diamond) soil temperature at 5cm sampled every 3 minutes at the open site,

wood site and cloche and summarised over monthly intervals from 28th September 2009 to 28th February 2010. b) The mean percentage (±SE) of *C. vicina* in diapause at the open (N=42), wood (N=45) and cloche sites (N=60) sampled between 28th October and 28th February. Adult cultures were established on 14th September 2009, eggs oviposited on 28th September and percentage diapause assessed on 28th October; and then every month thereafter.

Figure 5.8: October 2009 trial a) The maximum (red square), minimum (green triangle) and mean (blue diamond) soil temperature at 5cm sampled every 3 minutes at the open site wood site and cloche and summarised over monthly intervals from 2nd November 2009 to 2nd March 2010. b) The mean percentage (±SE) of *C. vicina* in diapause at the open (N=51), wood (N=36) and cloche sites (N=60) sampled between 2nd December and 2nd March. Adult cultures were established on 9th October 2009, eggs oviposited on 2nd November and percentage diapause assessed on 2nd December; and then every month thereafter. ^ designates where samples could not be recorded due to loss of larvae through predation. * designates where data was not recorded.

Figure 5.9: August 2010 trial a) The maximum (red square), minimum (green triangle) and mean (blue diamond) soil temperature at 5cm sampled every 3 minutes at the open site, wood site and cloche and summarised over monthly intervals from 14th September 2010 to 14th February 2011. b) The mean percentage (±SE) of *C. vicina* in diapause at the open (N=125), wood (N=192) and cloche sites (N=161) sampled between 14th October 2010 and 14th February 2011. Adult cultures were established on 28th August 2010, eggs oviposited on 14th September and percentage diapause assessed on 14th October; and then every month thereafter.

Figure 5.10: September 2010 trial a) The maximum (red square), minimum (green triangle) and mean (blue diamond) soil temperature at 5cm sampled every 3 minutes at the open site wood site and cloche and summarised over monthly intervals from the 2nd October 2010 to the 1st March 2011. b) The mean percentage (±SE) of *C. vicina* in diapause at the open (N=176), wood (N=156) and cloche (N=138) sites sampled between the 2nd November 2010 and the 1st March 2011. Adult cultures were established on the 14th September 2010, eggs oviposited on the 2nd October and percentage diapause assessed on 1st November; and then every month thereafter.

Figure 5.11: Mean (±SE) number of days for 50% of larvae (5 replicates of n=10) to pupate at a range of temperatures. Each datum point is based on 5 replicates of groups of 10 larvae. * designated samples which are not significantly different to each other.

Figure 5.12. The number of days (±SE) that L3 DO *C. vicina* took to reach 50% pupariation, after experiencing a defined period at three different elevated temperatures (n=60 for all data points). a) Adults held at 20°C, subsequent L3 larvae were then held at an elevated temperature of 20°C. b) Adults held at 20°C (dark grey) or 15°C (light grey), subsequent L3 larvae were then held at an elevated temperature of 20°C c) Adults held at 20°C (dotted) or 15°C (dashed), subsequent L3 larvae held at an elevated temperature of 15°C. All larvae were at D0 of diapause when placed at the elevated temperature and were held at 11°C before and after the period of elevated temperature. All adults were placed in a diapause inducing photoperiod (12:12, L:D). Groups labeled with * indicate that the group is significantly different to the control.

Figure 5.13. The number of days (±SE) that L3 D10 *C. vicina* took to reach 50% pupariation after experiencing a defined period at two different elevated temperatures 15°C (n=30) and 20°C (n=60). Larval progeny of 20°C adults, held at an elevated temperature of 20°C (dark grey) or 15°C (light grey) and subsequently held at 11°C after the period of elevated temperature. Groups labeled with * indicate that the group is significantly different to the control.

Figure 5.14. LT_{50} (days) (±95% fiducial limits) of (a) non-diapause (ND) and (b) diapause (D) third instar (L3) larvae exposed to -7°C. Larvae were progeny of adults reared at either 20°C (light grey), or 15°C (dark grey) then maintained at either 11, 5 or 0°C for 7 days prior to stress exposure. Exposure of the ND or D larval progeny of 15 degree adults to -7°C was not conducted.

Figure 5.15. LT_{50} (days) (±95% fiducial limits) for diapause (D) third instar (L3) larvae held -7°C at either DO (30d post oviposition), D10 (40d), D20 (50d) or D30 (50d) of diapause. Larvae are progeny of adults raised at either 20°C (light grey), or 15°C (dark grey). The LT_{50} of D30 larvae from 15°C adults was not recorded in this analysis.

Figure 6.1. Sample spectra from 2.3 to 4.6ppm. Spectra has been subjected to zero-filling, line broadening, Fourier transformation, phase correction, chemical shift referencing, removal of unwanted regions, normalization of total spectral area and then binning to 0.005 ppm. Mean values of all non-diapause samples are shown in green, mean values of diapause samples (excluding sample 17) are shown in blue. Examples of metabolite fractions: a-phenylalanine, b-glutamine, c-glycine, d- betaine, e- succinic acid.

Figure 6.2. PC1/PC2 scores plot for PCA of *C. vicina* larvae pJRES spectra with D samples (red triangles) and ND samples (green squares). D sample #17 can be seen as a clear outlier. Technical replicates are shown as a grey diamond.

Figure 6.3 a) Repetition of pJRES3 with the exclusion of sample D17. The PC1/PC2 scores plot for the PCA of the blowfly larvae pJRES(3) spectra with diapause samples (red triangles) and non-diapause samples (green diamonds). Figure 6.3**b)** shows the loadings plot illustrating key peaks leading to the differences noted in PC1.

Figure 6.4. Comparison of the 34 metabolites identified in 20 non-diapause or 19 diapause *C. vicina* L3 larval samples after ^{1}H NMR analysis. Quotients of mean content of diapause (D) over non-diapause (ND) were plotted (i.e. fold change). Red bars represent metabolites that were more abundant in D larvae; blue bars represent metabolites more abundant in ND. * designates metabolites only seen in one condition (D or ND). ^ designates metabolite significant to P<0.001. ~ designates metabolite significant to P<0.05.

Figure 7.1. Schematic depiction of the effect of climate warming upon diapause. The lower multi-colored line represents current transition through stages of development (green), diapause (red) and post-diapause quiescence (yellow). The thick arrow represents the time of year at which *C. vicina* (at 52°N) currently experiences its critical day length (CDL) (around 5th September). Because diapause initiation in the majority of *C. vicina* can only occur if mean soil temperature remains below 15°C, an increase in temperature may lead to a delay in entrance into diapause or complete diapause aversion. This is represented by the upper multi-coloured line, as is an early termination of diapause and a shorter post-diapause quiescence. Figure modified from Hayward *et al*., unpublished.

List of Tables

1. Introduction

With over a million described species, the class of Insecta has greater diversity than all other classes of living organisms. The exact number of species of insect is not known, but there have been estimates of between 4 million (Novotny *et al.*, 2002) and 30 million (Erwin, 1982), with the most recent estimation being at the lower end of this scale. The highest diversity is typically found around the equator, with a general trend of decreasing diversity with increasing latitude. Insects are found in nearly all environments in the world, from the Antarctic springtail, *Gomphiocephalus hodgson,* on the Antarctic continent (Sinclair & Sjursen, 2001), the goliath beetle, *Goliathus giganteus*, of the African tropical forests, to the Ocean skater, *Halobates germanus,* which lives on the Indian and Pacific ocean (Pruthi, 1932). Insects provide a vital role in keeping ecosystems functioning; they are the pollinators of plants, decomposers of organic material, producers of silk, aerators of soil and controllers of plant pests. So vital is the role of insects in the food chain that it is thought that if insects were all to disappear then most other life forms (reptiles, birds and mammals) would be extinct in a matter of months (Wilson, 1992).

For insects inhabiting temporal, boreal or polar latitudes, winter poses a significant threat to survival. As ectotherms, insect body temperatures are closely related to environmental temperature, so prolonged periods at low temperature or freezing can both have deleterious effects on fitness and ultimately survival. Even when temperatures are not prohibitively low for survival, winter often results in limited food sources, osmotic stress due to frozen ambient water, and decreased activity levels. In response to this seasonal transition, some insects migrate to a warmer climate but many remain *in situ* and enter an environmentally adaptive dormancy termed diapause.

There are two main strategies to respond environmental stress. Firstly, if an aseasonal decrease in temperature, moisture or food availability occurs insects, can enter a reversible state of

suppressed metabolism termed quiescence, which generally requires no long term physiological preparation. The alternative is diapause, which forms the major dormancy strategy that insects can use to respond to seasonal predictable deterioration in habitat, this dormancy can be seen in winter (hibernal), or summer (aestival). Diapause differs from quiescence in some key respects. Diapause is a multifaceted preemptive tool used to survive unfavourable conditions; it usually precedes adverse conditions, and does not necessarily end when favourable conditions recommence (Bale & Hayward, 2010). Quiescence is an immediate response to adverse environmental factors below species-specific thresholds, development can be resumed immediately once ideal conditions are experienced (Kostal, 2006). Aestivation is a multifaceted tool employed by insects, much like diapause, but it allows insects to survive high temperatures; it is also known as 'summer diapause' (Liu *et al.*, 2006), 'tropical diapause' (Canzano *et al.,* 2006) or 'hot thermal diapause' (Nibouche, 1998).

1.1. Diapause

Diapause is used as a means to survive seasonally predictable unfavourable environmental conditions, such as temperature extremes, drought or reduced food availability. The key feature of diapause is its anticipatory nature, in that it begins before the onset of unfavourable conditions, either because it is genetically predetermined (obligatory diapause), or induced by other environmental cues (facultative diapause). Its onset, maintenance and termination are characterised by a number of morphological, behavioural, physiological and biochemical features (Tauber *et al*., 1986). Diapause typically confers low metabolic activity (Kostal, 2006), low morphogenesis (Lopez *et al.* 1995), increased resistance to environmental extremes (Hayward & Saunders, 1998) and altered behavioural activity (Kostal, 2006), and has very specific triggering (McWatters & Saunders, 1996) and releasing conditions (Denlinger, 1972). Diapause also synchronizes the insect's life cycle with seasonal changes in its environment, including winter conditions (Bale & Hayward, 2010). Diapause usually occurs during a specific

season and at a specific predetermined stage of development for each species (Kostal, 2006). Most insects enter diapause during just one stage of development, but some species with long life cycles may enter diapause during more than one stage in more than one season. For example, the cockroach *Ectobius lapponicus* can diapause either as an egg or as a 4^{th} instar nymph (Brown, 1973).

1.1.1. Obligatory and facultative diapause

There are two types of diapause, facultative and obligate. Obligate diapause is less commonly observed; the developmental arrest is fixed in every individual and is solely under the control of genetics. As such, it occurs for every individual of the population at a set life stage, regardless of the environmental conditions experienced. One example of an insect which experiences an obligate diapause is the solitary bee, *Osmia lignaria* (Sgolastra *et al.,* 2010); one month old cocooned, unfed adults decreased their respiration rate to 0.1 $ml.g^{-1}.h^{-1}$ regardless of rearing temperature. Facultative diapause, however, takes cues from environmental conditions, and as such will only occur in specific life stages if the environmental cues are correct. The bark beetle, *Ips typographus,* (Dolezal & Sehnal, 2007) is one example of an insect with a facultative diapause. Photoperiod and temperature form the key regulators of diapause in *I. typographus,* with a photoperiod less than 14.7 hours resulting in 50% diapause incidence (Dolezal & Sehnal, 2007). This photoperiod, inducing 50% of a population to enter diapause, is termed the critical day length (CDL), and varies depending on the geographic location. For example, different latitudinal strains of the blue bottle fly, *Calliphora vicina*, have different CDLs, with longer photoperiods required to induce diapause at higher latitudes (McWatters & Saunders, 1998).

The diapause syndrome is split into three main phases: pre-diapause, diapause, and post-diapause (Kostal, 2006). The pre-diapause phase breaks down into two sub-phases: induction

and preparation. Diapause stage contains three sub phases: initiation, maintenance and termination. This is then followed by post-diapause quiescence (fig. 1.1).

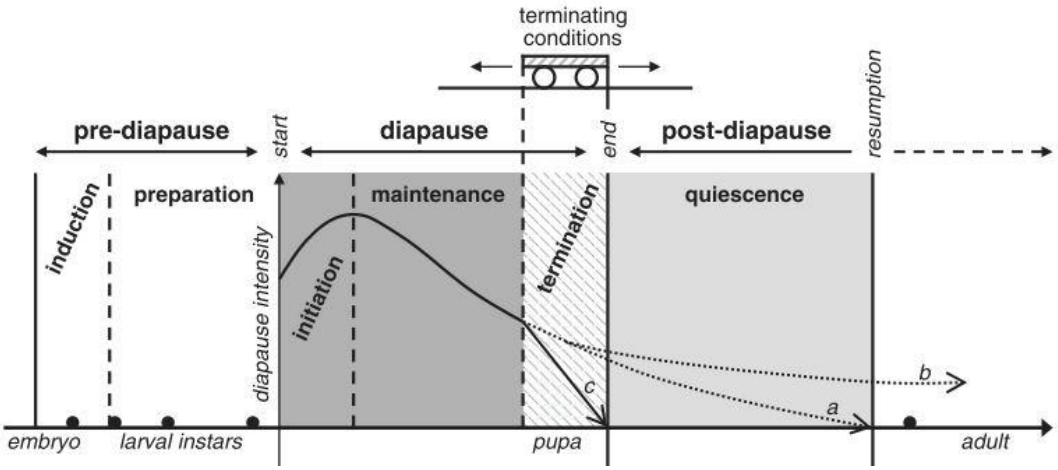


Figure 1.1. Schematic depiction of the three main diapause stages, pre-diapause, diapause and post-diapause. Further sub-phases are indicated by vertical dashed lines, namely, induction, preparation, initiation, maintenance, termination and quiescence. A thick line with arrowhead indicates the passage of time from zygote, through to the end of diapause. Some species may omit certain stages, this is species specific. Diapause intensity is also species specific and can be affected by environmental conditions, as noted in the three schematic dotted lines, a), b) and c). Lines a) and b) represent conditions where environmental conditions are unfavourable for further development, c) represents favourable environmental conditions. Figure modified from Kostal, (2006).

1.1.2. Pre-diapause stage

The primary feature of diapause is its anticipatory nature; environmental cues are perceived by the insect and stored, then at a later stage the stored information is translated into neuroendocrine functions, which start diapause induction. The duration over which the information is stored can span a number of developmental stages, or even generations. For instance, larvae of the zygaenid moth, *Tyrassia penange*, are sensitive to photoperiod, with a CDL of 13.5 hours (h); when larvae experience a photoperiod shorter than the CDL, diapause occurs in the prepupal stage (He *et al*., 2009). The northern tamarisk beetle, *Diorhabda carinulata* has both its sensitive stage and diapause stage as an adult beetle, responding strongly to photoperiod, with a CDL of 14h 39min (Bean *et al*., 2007a, b).

1.1.3. Induction

Diapause is induced in advance of adverse environmental conditions, i.e. insects respond to token stimuli to cue diapause rather than the direct effects of adverse weather. The stage that the token stimuli are received, the sensitive stage, is genetically determined and is species specific (see table 1.1). Once token stimuli have been received, neuroendocrine, metabolic, morphological and behavioural changes can occur within, though in some species (discussed below) this can be reversed if conditions do not remain ideal.

Table 1.1. The sensitive life stage of insect species, and the corresponding diapause stage.

Species	Sensitive stage	Diapause stage	Reference
Diatraea grandiosella	Early larval	Late larval	Gadenne *et al*., 1997
Sarcophaga crassipalpis	Maternal adult	Pupal	Rinehart *et al.,* 2000
Manduca sexta	Late embryonic	Pupal	Kingslover & Nagle, 2007
Bombyx mori	Parental adult	Egg	Saravanakumar *et al*., 2008
Lymantria dispar	Late embryonic	First instar	Atay-Kadiri & Benhsain, 2005
Calliphora vicina	Maternal adult	Third instar	McWatters & Saunders, 1996
Lucilia sericata	Parental adult	Third instar	Tachibana & Numata, 2004

1.1.4. Preparation

Diapause preparation typically occurs in insects which have an extended amount of time between induction and diapause initiation; whereas some species, in which the sensitive stage and the diapause stage are close together, do not enter a preparation stage. During the diapause preparation stage, the information that was cued during diapause induction is 'stored'. Diapause preparation is important in some species as it can influence physiological and behavioural activities such as migration (Dockx, 2012), selection of a suitable habitat for overwintering (Brodeur & McNeil, 1990), aggregation (Schwarz & Gries, 2010) or the building up of the quantity and quality energy reserves required before diapause (Hahn & Denlinger, 2007). The increase of energy reserves is important as the majority of diapausing insects do not feed until diapause has ended (Hahn & Denlinger, 2007). Some diapause-destined insects, such as the Indian meal moth, *Plodia interpunctella,* increase their body weight by up to 45% in preparation for diapause (Tsuji, 1958). Not all insects increase their energy reserves, however with third

instar larvae (L3) of the blow fly, *C. vicina,* (Saunders, 1997), and pupae of the tobacco hornworm, *Manduca sexta* (Siegert, 1986) remaining the same size whether programmed for diapause or not. The growth rate can also be modulated whilst preparing for diapause, although the growth rate is normally directly dependent on temperature (as insects are poikilotherms). One such example is the lady bug, *Epilachna vigintioctopunctata*, which was noted to decrease its growth rate while simultaneously increasing its feeding rate (Kono, 1980). It was suggested that *E. vigintioctopunctata* was able to selectively alter the rate of development to prevent early molting, when environmental temperatures would have been above those needed to allow diapause to progress.

1.1.5. Diapause stage

The diapause stage is associated with depressed morphological development and an alternate pathway of physiological events. The diapause state is split in to three sub-stages, initiation, maintenance and termination.

1.1.6. Diapause initiation

During the initiation (also known as beginning, fixation, entry, onset, start or intensification) stage, feeding is slowly suppressed. In some insects, feeding completely ceases and all gut contents are often evacuated (Lee & Denlinger, 1985; Woodman, 2012). Metabolism also decreases during the initiation stage, which can be shown by measuring oxygen consumption. For example, in the apple maggot fly, *Rhagoletis pomonella,* metabolic rate during the diapause stage is much lower than in non-diapause, pre-diapause (fig. 1.2a) or post-diapause (fig. 1.2b) stages (Ragland *et al.,* 2009). Metabolic activity can be further decreased by aggregation, for instance the firebug, *Pyrrhocoris apterus,* is able to significantly decrease energy metabolism from $0.052Wg^{-1}$, when held individually in diapause, to $0.033Wg^{-1}$ when aggregated in a group of 50 individuals (Su *et al.*, 2007). Other noted changes include decreased RNA synthesis

(Wigglesworth, 1972), altered sensitivity to injected hormones (Denlinger & Bradfield, 1980) and increased cold tolerance (Hodkova & Hodek, 2004; Kostal *et al*., 2004a, b).

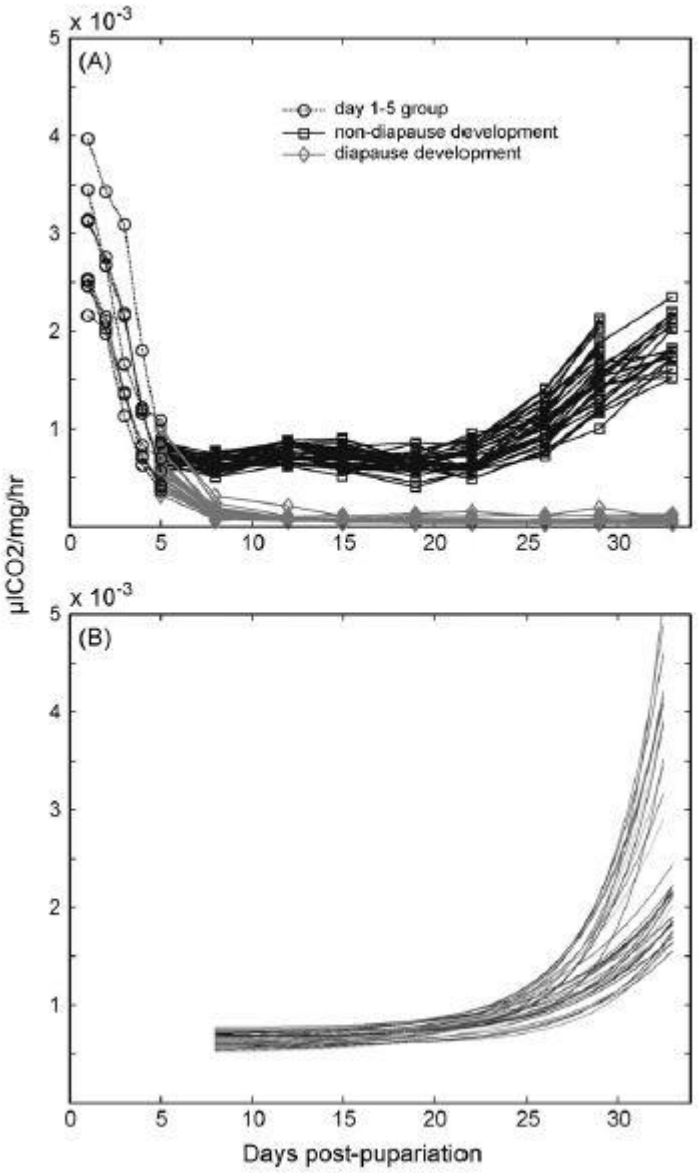


Figure 1.2. a) Metabolic rate of non-diapause (square) and diapause (diamond) pupa. Data for the first eight days are from a separate cohort (circles). b) Non-linear regression of metabolic rate of larvae resuming active development. Figure taken from Ragland *et al.*, (2009).

During the diapause initiation stage insects still may remain sensitive to environmental conditions (Tauber *et al.*, 1986). For example, temperature conditions during this period can provide an important role in determining the intensity of the diapause; this is seen in the locust, *Locust migratoria*, in which there is an increase in diapause intensity when incubated at 10^{o}C

compared to 35^{o}C (Ando, 1993). In most species, if the temperature is too high during this period, the diapause syndrome can be completely halted and insects can revert to a non-diapause pathway (Kimura & Minami, 1980). This trait is noted in *C. vicina* with larvae averting diapause if held above 12 to 17^{o}C, depending on the geographic strain (Vinogradova & Zinovjev, 1972). Intermediate temperatures can lead to a less intense diapause, which can affect cold hardiness. This decoupling characteristic is linked to seasonal polyhenism in some species, e.g. in the papilinoid butterfly, *Papilio polyxenes,* where colour is linked to photoperiod received. Butterflies experiencing a long day length are green in colour, while those that receive a short day length are brown and those that receive an intermediate day length are an intermediate colour (Hazel & West, 1983). Whilst in diapause insects often display increased cold tolerance characteristics (see chapter 1.3.3. for more details) if diapause is halted before the onset of adverse climatic conditions, insects can suffer increased mortality.

1.1.7. Diapause maintenance

Diapause is often maintained throughout environmental conditions that would allow direct development to continue. This is achieved by locking individuals into a developmental arrest through metabolic suppression to a constant low level; as such diapause can be maintained from a period of weeks to years. Although this stage remains the only 'proper' stage of diapause, it is the least researched in regards to its physiological nature. It is thought that throughout the maintenance stage, individuals slowly become more sensitive to diapause terminating conditions (Hodek, 1983; Sawyer *et al.,* 1993).

In a number of species two hormones have been found to regulate of diapause: juvenile hormone (JH) and prothoracicotropic hormone (PH). The hormonal regulation of diapause is stage and species specific, diapause in eggs is maintained by high titers of ecdysteriods; diapause will be maintained until the ecdysteroid titer drops (Lee & Denlinger, 1996). Larval diapause is maintained through a cessation of the brain-prothoracic gland axis; the prothoratic

glands are prevented from releasing ecdysteriods, which is either due to the low titer of PH or due to the prothoracic gland becoming unresponsive to PH (Meola & Adkisson, 1977; Richard & Saunders, 1987). PH is a small homodimeric brain peptide that has been identified in Lepidoptera, but is thought to "probably" be in other insects too; it is not only important in regulating diapause but recent research has suggested that it may have a direct role in the development of female accessory gland reservoirs and male accessory gland tubules, though the exact nature its use in these areas is not yet known (Rybczynski *et al*., 2009). Hormonal control of adult diapause can chiefly be attributed to a very low level of, or a complete absence of, JH (Denlinger, 1985). Activation of the *corpus allatum* will increase levels of JH and lead to the termination of diapause. JH (previously known as neotenin) is a sesquiterpenoid hormone, there are four homologs, JH 0, I, and II are found in Lepidoptera and JH III is found in all insects and is thought to be the evolutionary precursor (Edwards *et al*. 1995).

In some species, an actual diapause hormone (DH) has been identified (Gullan & Cranston, 2005). Perhaps the best studied of these is the silk moth *Bombyx mori*, in which DH is released from the adult suboesophageal ganglion to initiate an embryonic diapause (Iwata *et al.,* 2005), The DH induces expression of the trehalase in ovaries, which is responsible for the accumulation of glycogen in the egg which is then converted in to sorbitol (Yamashita & Hasegawa, 1985). *In vitro* experiments conducted by Iwata *et al*. (2005) showed that the sorbitol inhibited the development of diapause embryos. DN is a neuropeptide which contains a C-terminal pentapeptide homologous to pheromone biosynthesis activating neuropeptides (PBAN's) which have a diverse array of uses including melanization in moth larvae (Uehara *et al.,* 2011), contraction of visceral muscle in cockroaches (Predel, 2001) and a reduction of trail pheromone in fire ants (Choi & Meer, 2012)

1.1.8. Diapause termination

Much like the other stages of diapause, the process of diapause termination is often species specific. For example, the pitcher plant mosquito *Wyeomyia smithii* are sensitive to photoperiod as a means of diapause termination, with a photophase of 16.6h (of light) causing the termination of diapause (Emerson *et al*., 2010). Chilling is also known to cause diapause termination, e.g. in the parasitoid, *Colpoclypeus florus*, in which a chilling period of >5 weeks is required for termination (Milonas & Savopoulou-Soultani, 2000). The cercropia moth, *Hyalophora cecrropia,* terminated diapause due to a sudden drop in temperature (Williams, 1956) but it is not known whether this occurs in the field. Other species do not uniformly rely on any one environmental factor to terminate diapause, nor does termination occur as soon as favourable conditions are encountered by the insect (Tauber *et al*., 1986). This is due to a series of specific developmental processes that must occur, usually in a specific order, before diapause can be terminated (Veerman, 1992). It is the case with many autumn diapausing insects that they gradually lose sensitivity to diapause maintaining factors, after which spontaneous termination usually occurs some time during late autumn or during winter. It has also been proposed that diapause termination, as with other stages of development, is calculated using accumulated day degrees, as was seen in Saturniids (Mansingh & Smallman, 1971) and the tortricid moth, *Adoxophyes orana,* which terminated diapause after 418 day-degrees (Milonas & Savopoulou-Soultani, 2006). However, this view is not widely agreed upon in most species. Due to this complex linkage of factors and the paucity of data, it is not yet currently known how adaptive each of these triggers are to environmental cues. It is thought that diapause termination is cued by both internal and external factors, and as such occurs over a broad period of time. Insects then enter the post-diapause stage.

1.1.9. Post diapause

Unless environmental conditions are unsuitable, insects will continue direct development when diapause ends. If conditions are not suitable, i.e. temperature is below the developmental threshold, then insects can enter a state of suppressed development termed post-diapause quiescence (PDQ). For instance, the flesh fly, *Sarcophaga bullata*, terminates diapause (at 40^{o}N) in January, but remains in PDQ until prevailing environmental conditions allow direct development to continue, typically in early April (Denlinger, 1972). The notable difference between quiescence and diapause is that once favourable conditions return, insects in quiescence can immediately resume development, whereas individuals in diapause must complete diapause termination processes before development can resume (Tauber *et al*., 1986). There is no set length of time for PDQ, and it should also be noted that not all insects enter this stage. There are disadvantages to being in a post-diapause quiescent state, as cold hardiness and drought tolerance may be reduced in some species, e.g. the predatory mite, *Euseius (Ambuyseius) finlandicus* (Broufas & Koveos, 2001). In other species, however, stress tolerance mechanisms do persist into PDQ. For example, *S. crassipalpis* was seen to sustain diapause-like expression of Heat Shock Proteins (HSPs) until direct development was initiated (Hayward *et al*., 2005).

Another key role for PDQ is to synchronise insect emergence in spring across populations that may have had staggered entry into diapause. As diapause ends over a broad period of time, the quiescent stage may act to synchronise insects by allowing them to simultaneously resume active development when the environmental conditions exceed the developmental threshold.

1.2.Alternate diapause durations

1.2.1. Fixed prolonged diapause

Fixed diapause is where insects in the population will emerge after a fixed duration but not before or after that fixed duration, this can vary as there are often different genotypes within a population, i.e. variability within a population without plasticity in individuals (Menu, 1993). A fixed diapause may lead to a reduction in the individual's fitness due to: an increase in mortality due to an increased duration of diapause, loss of reproduction opportunities due to remaining in diapause when conditions are favourable for development or terminating diapause in unsuitable conditions for development which may lead to decreased fitness and increased mortality (Menu & Debouzie, 1993). Though there are advantages to entering a fixed diapause as was seen in the two year diapause cycle in eight species of *Xestia* moths in Sweden, which seen to reduce parasitism due by the univoltine parasitic wasp, *Ophion luteus,* by showing high synchrony in their two year diapause and therefore most *Xestia* were seen to emerge in every other year, thus reducing the numbers of *O. luteus* (Varkonji *et al*., 2002).

1.2.2. Bet hedging within the population

Risk spreading is common among insects and plants that experience fluctuations that can impact on fitness. The modeling of risk spreading is well established within seed germination of plants (Cohen, 1966; MacArthur, 1972; Venable & Lawlor, 1980). In Cohen's (1966) model of bet-hedging three predictions were given, firstly if seeds did not germinate in an unsuitable year would germinate under the same conditions the following year, secondly that the proportion of germinating seeds would be positively correlated with how favourable the conditions were. Finally the production of seeds that could germinate over a number of years would be produced by all individuals. These strategies can easily be applied to diapause (Tauber et al., 1986; Hanski, 1988). The advantages of bet-hedging within diapause are that a female can increase her fitness

as her offspring could be spread over many years (depending on the species), as such there is a greater chance that some of her offspring would experience ideal conditions, which would allow the continuation of her genes. Conversely, it could lead to a decrease in fitness due lost reproduction opportunities and in increased chance of predation with the longer that an insect spends in diapause and potential death before emergence due to diminished energy resources. Cohen's (1996) model does appear to be applicable to insect diapause in some species. The desert ground-nesting bee, *Perdita portalis,* is one such example, in which 73% of larvae terminated diapause after one year, a high proportion of those remaining pupated in the second year (93%) and the remainder pupated in the third season (Danforth, 1999). Another example is the European chestnut weevil, *Curculio elephas,* after one or two years in diapause 95% will emerge, with the rest emerging in the following two years (Soula & Menu, 2003). There has also been accounts of insects terminating diapause if future conditions are thought to be suitable, Hanski (1989) and Roques (1989) found that the number of insects that terminated diapause was higher in years that went on to have a higher cone or seed crop.

1.2.3. Tropical diapause

As mentioned earlier, diapause is not only an overwintering strategy, but can also occur in the tropics. In such cases, it is often employed as a strategy to avoid adverse biotic factors (e.g. food resources), rather than abiotic factors (Denlinger, 1986). For example, food for necrotrophic insects may be more abundant following dry seasons, or oviposition sites (e.g. fallen trees) may be more abundant during rainy seasons. Thus, the primary function of diapause in many tropical species may be to synchronize mating seasons or competition reduction strategies, rather than to avoid harsh environmental conditions. Like winter diapause, tropical diapause is anticipatory, i.e. it is induced before a habitat dries out or is flooded; the food resource becomes limited/changes in quality; or the activities of competitors/predators increase (Masaki, 1980;

Denlinger, 1986; Godfrey & Hassell, 1987; Wolda, 1988; Topp, 1990; Tanaka, 2000; Adis & Junk, 2002).

Diapause in tropical climates is faced by several challenges that temperate diapause is not. Metabolism is often reduced in temperate zones with the aid of low temperatures; however, tropical species have to reduce their metabolism whilst remaining at high temperatures (Denlinger, 1986). Linked to this is dehydration, as during diapause, most water is lost through the spiracles as a result of respiration, rather than through the cuticle (Zachariassen & Maloiy, 1989). Insects can selectively close their respiratory spiracles to reduce water loss, and this is noted as a prominent feature at higher temperatures (Terblanche *et al*. 2010). For example, in the hessian fly, *Mayetiola destructor*, pupae are able to decrease their transpiration rate from 0.47% per hour in non-aestivating pupae to 0.28% per hour during aestivation, this rate was further decreased as aestivation progressed (Benoit *et al*., 2010a). Another challenge that tropical diapausing insects face is maintaining an active immune system. Temperate insects can usually afford to depress their immune system as the low ambient temperatures are not conducive to bacterial and fungal growth. Although insects are known to depress their immune system over winter, Marshall & Sinclair (2010) noted that the woolly bear caterpillar, *Pyrrharctica Isabella,* is able to enhance its immune system after freezing, this may be to prevent fungi / bacteria spreading through any injury sustained through freezing. Finally, in temperate climates, predators often either migrate or hibernate, whereas in tropical climates the risk of predation is still high (Denlinger, 1986).

1.2.4. The role of diapauses in regulating phenology

Phenology is thought to affect nearly every aspect of the ecology and evolution of every species (Forrest & Miller-Rushing, 2010). Nearly all biological phenomena have annual cycles, and these are known to be influenced by the timing of annual events. For insects, the relationship between phenology and life history is paramount, as the processes which underlie phenological

adaptations are not static; therefore, insects are able to modulate their phenology in response to natural situations. Knowledge of an insect's phenology is critical for understanding population growth, migration, speciation and extinction (Tauber & Tauber, 1976).

1.3. Insect cold tolerance

Most temperate insects have to employ strategies to survive at low temperatures, which fall into two primary categories: freeze avoiding (FA) or freeze tolerant (FT) insects (Salt, 1961). The principal difference between freeze tolerance and freeze avoidance is the ability of FT species to survive internal (but extracellular) ice formation. For FA insects, any internal ice is lethal. The temperature at which spontaneous freezing occurs is termed the supercooling point (SCP), or temperature of crystallization (Zachariassen, 1985). Within the laboratory this can easily be identified due to the exotherm produced by the release of the latent heat of crystallization (fig. 1.3). In brief, the insect is attached to a thermocouple and then placed inside a controlled temperature alcohol bath; the alcohol (and insect held within) is gradually cooled; when the insect freezes, an exotherm will be recorded on the output (fig. 1.3). Any species which survive after reaching their SCP are classified as FT, while those that die are classified as FA.

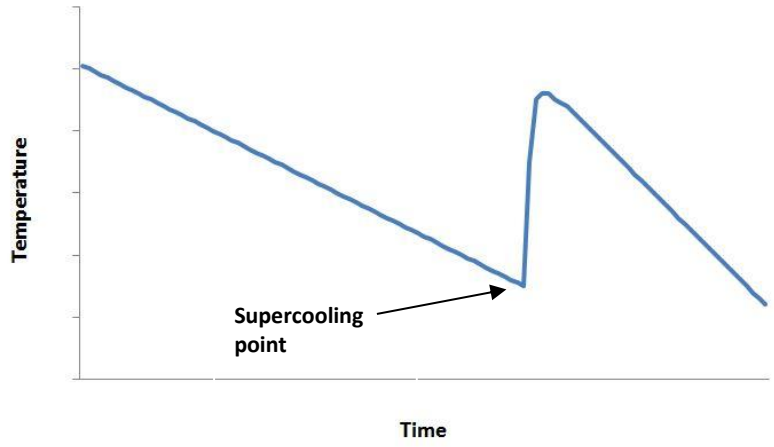


Figure 1.3. Schematic of freezing in insects. The blue line represents body temperature as it is cooled over time; the exotherm (point of freezing) is labelled.

1.3.1. Freeze tolerance vs. Freeze avoidance

The mechanisms underpinning FT and FA differ and are discussed below (fig. 1.4).

Freeze tolerant insects have adapted to survive the formation of ice crystals in extracellular spaces to allow survival below their SCP. FA insects are more common than FT insects in temperate regions of the northern hemisphere, though this is reversed in the southern hemisphere, where FT insects dominate (Sinclair & Chown, 2005). Sinclair *et al.* (2003) suggested that the evolution of this trait was convergent, evolving separately in both the southern and northern hemispheres.

Extracellular freezing normally occurs prior to intracellular freezing, as intracellular freezing may cause rupture of the cell membranes, which would lead to cell death. Extracellular freezing is initiated by ice nucleating agents (INAs) in the haemolymph; these are molecules which promote ice formation (see section1.5.3). Freeze tolerant species also accumulate polyhydroxyl alcohols (polyols) and sugars, which help to slow the rate of ice formation and stabilize protein structures, which can be damaged due to low temperatures (see section 1.5.1). Antifreeze proteins are also produced which in FT species are thought to increase survival by controlling extracellular freezing and preventing recrystallisation (see chapter 1.5.2).

The majority of temperate insects in the northern hemisphere rely on supercooling for survival at low temperatures (Leather *et al.*, 1993). FA and FT species differ in some key strategies, firstly, after choosing a suitable overwintering site, FA insects will stop feeding and evacuate all gut contents, as the gut contents often can act as ice nucleators, as was noted in the fire-colored beetle larvae, *Dendroides canadensis* (Olsen & Duman, 1997). Freeze avoiding species then accumulate polyols and sugars, which act to depress the SCP of the insect (Hodkova & Hodek, 1997). As with FT insects, AFP's are subsequently produced (Bennett *et al*., 2005), (see section 1.4.3 for more details).

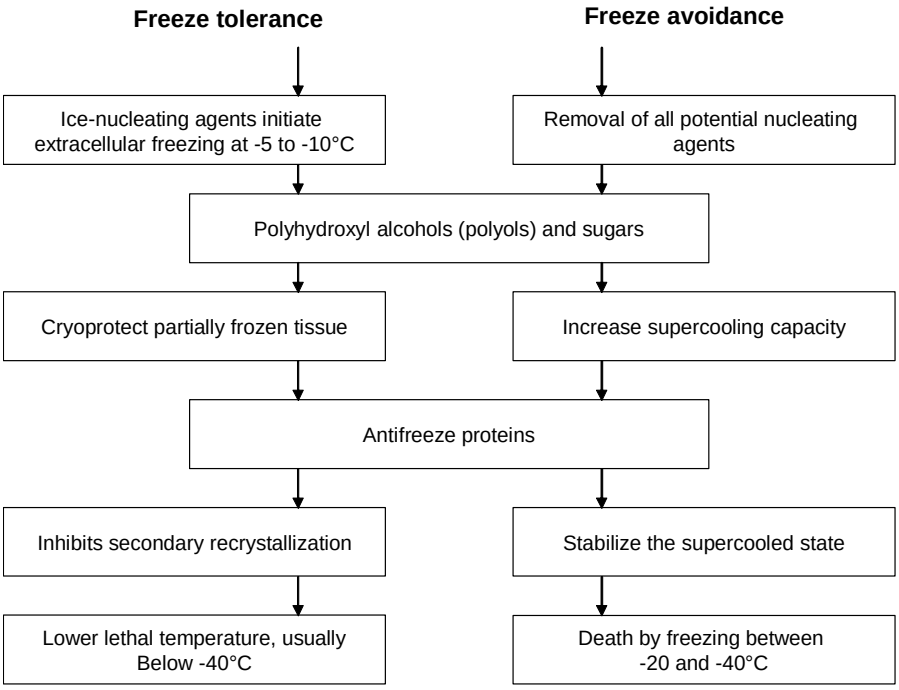


Figure 1.4. Summary of biochemical mechanisms of freeze tolerant and freeze avoiding insects (Bale, 1996, 2002).

1.3.2. Problems with Salt's definition

Salt defined all survival based on the ability to tolerate freezing, but as an increasing number of species were examined, it was noted that there was a broad spectrum of pre-freeze mortality (fig. 1.5; Knight, 1987; Bale *et al.*, 1988). Bale (1996) suggested three additional categories: chill tolerant, chill susceptible and opportunistic survival. Not only do different species have different abilities to tolerate cold, but the cold hardiness within life stages of a species is known to dramatically differ (Leather *et al.,* 1993).

- Chill-tolerant insects have a low SCP but show mortality at temperature above the SCP. For example overwintering, adults of the beech weevil, *Rynchaenus fagi*, can extensively supercool and have a SCP of c. -25^{o}C in mid-winter, though 74% of the population died if held at -15^{o}C for 50 days (Bale, 1991).
- Chill-susceptible insects have a low SCP but show very high mortality at temperature above the SCP. One such example of a chill-susceptible insect is the overwintering aphid, *Myzus persicae,*

which has a SCP of -25°C, and displays high mortality when held at temperatures between 7-10°C for just one minute (Clough *et al.*, 1990; Howling *et al.*, 1994).

- Opportunistic-survival insects are unable to survive if they experience temperatures which are lower than the threshold temperature for the insects development, as such insects must find a sheltered site for successful overwintering. Overwintering houseflies, *Musca domestica*, have to seek sheltered overwintering sites due pupae displaying 0% survival at 0°C for 5 days, despite having an SCP -15°C (Coulson & Bale, 1991).

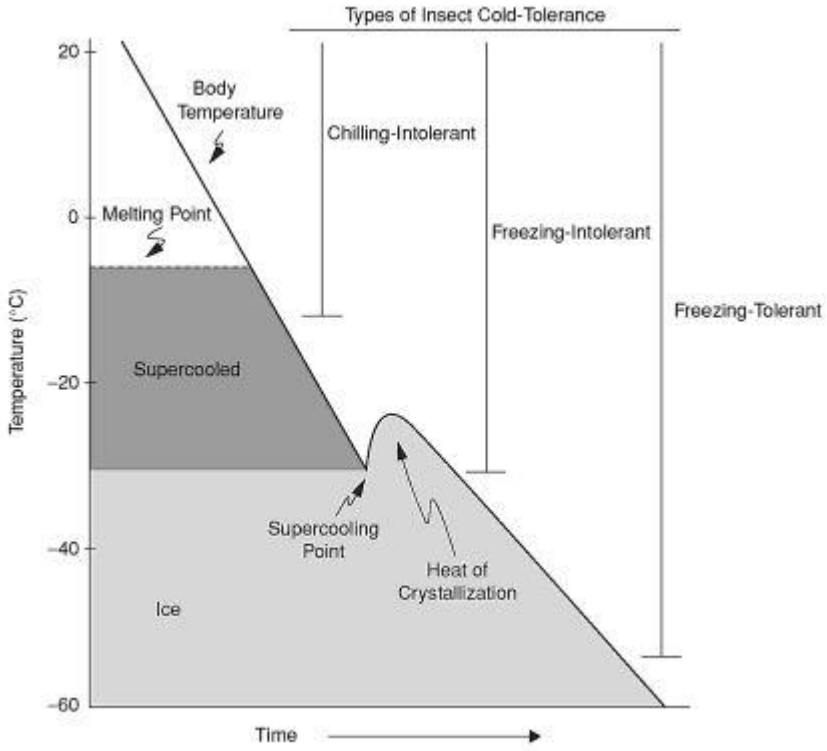


Figure 1.5. Schematic of freezing in insects. The bold line represents body temperature, below is outlines what state body fluids are in. When the insects body temperature decreases below its supercooling capacity it will freeze (reach the supercooling point), temperature will then rise as heat is released due to crystallization of body water. Bars on the top right indicate ranges for chill-tolerant, freeze-avoiding and freeze tolerant insects. Figure taken from Michaud & Denlinger (2010).

1.3.3. Cold tolerance and diapause

There remains debate regarding whether enhanced cold tolerance is an inherent part of the diapause programme in all insects (Pullin, 1996; Denlinger, 2002; Vesala & Hoikkala, 2011). For

some species, successful cold acclimation and overwintering appear to be reliant on individuals entering diapause (Denlinger, 1991; Slachta *et al*., 2002). For example, diapausing females of the two-spotted spider mite, *Tetranychus urticae,* had a lower lethal temperature at which 50% of the population suffer mortality ($LTemp_{50}$) of -19.7°C, compared to non-diapausing females (-13.3°C) (Khodayari *et al*., 2012). Cold tolerance is also increased as diapause progresses in the pistachio fruit hull borer, *Arimania comaroffi,* in which non-diapause pupae showed little survival (7.5%) when held at -10°C for 24 hours, while diapausing pupae in October had a survival rate of 25.5%, which increased month on month until January, to a maximum of 67.3% (Bemani *et al.*, 2012). For other species, however, cold tolerance can be increased as winter progresses, independently of diapause (Young & Block, 1980; Fields *et al*., 1998). For instance, the $LTemp_{50}$ of the cotton bollworm, *Helicoverpa zea*, was not significantly different in diapause and non-diapause pupae (Morey *et al*., 2012). Acclimation is also known to further increase cold hardiness within diapause. For instance, diapausing larvae of the codling moth, *Cydia pomonella*, when exposed to 5°C, experienced a 77% survival rate, yet when acclimated 96% of larvae survived (Khani *et al*., 2007). An increase in cold tolerance due to acclimation was also seen in diapause eggs of the migratory locust, *Locusta migratoria,* in which individuals exposed to -10°C for 3 days experienced 41.1% survival, while those acclimated for 10 days at 5°C had 83.3% survival at -10°C (Jing & Kang, 2003). This acclimation effect is not unique to diapause however, and there are many examples of how acclimation also enhances the cold tolerance of non-diapause insects. For example, non-diapause eggs of the yellow-spotted longicorn beetle, *Psacothea hilaris,* showed less than 30% survival when held at -22°C for 2h, though when acclimated at 0°C for 4h prior to exposure to -22°C survival increased to c. 60% (Shintani & Ishikawa, 2007).

1.4. Measuring insect thermal tolerance

There are a number of recognised methods of assessing thermal tolerance, including super cooling point (also known as the temperature of crystallization, T_c), lethal time, lethal temperature and discriminating temperature.

1.4.1. SCP

As discussed earlier, the SCP of an insect is the temperature at which spontaneous freezing of body water occurs (Zachariassen, 1985), which is indicated by the exotherm produced, caused by the release of the latent heat of crystallization (fig. 1.3). In brief, the insect is attached to a thermocouple and then placed inside a controlled temperature alcohol bath, using a set ramping rate (usually between 1°C and 0.1°C.min^{-1}) the alcohol (and insect held within) is cooled, when the insect freezes an exotherm will be recorded on the output (fig. 1.3). The rate of cooling is important as, particularly in large bodied insects, the rate must be such that the whole insect is cooled at an equal rate (i.e. small insects can be cooled quicker as they have a smaller volume and core temperature is most likely to mirror exterior temperature), a slower rate is also more ecologically realistic (Kelty & Lee, 1999). Varying the cooling rate can affect the SCP (Salt, 1961). For example, in the sub-Antarctic springtail, *Tullbergia antarctica,* the mean SCP increased with decreasing cooling rate (from 8 to 0.1°C.min^{-1}), as the cooling rate was further slowed (from 0.1 to 0.025°C.min^{-1}), the SCP declined from -6 to <-12°C (Worland, 2005).

It should be noted that the SCP cannot be used as a sole classifier of cold tolerance, as many FA insects suffer pre-freeze mortality before they reach their SCP, and FT insects often survive temperatures well below their SCP (Chen *et al.,* 1991; Nedved, 1993; Somme, 1996; Hatherly, 2004). As such it should only be used to classify if insects are freeze tolerant/avoiding or should be combined with other indices of cold tolerance to determine a true representation of cold hardiness.

1.4.2. Lethal time and temperatures

The lethal time (LTime) is the time at a set temperature taken for a specific percentage of a group of insects to be killed. Common indices of LTime are $LTime_{10, 50, 90}$, this being the time at a given temperature for 10%, 50% and 90% mortality to occur. For lethal time experiments, insects are held at a controlled temperature for different amounts of time before being returned to their original temperature. Probit analysis can be carried out to linearise the sigmoidal curve found in lethal time, lethal temperature and lethal dose (LD) experiments (Finney, 1971; Fry, 1993), this enables an accurate estimation of the $LTime_{50}$. This method of analysis has been utilised in other studies (Hart *et al*., 2002; Hatherly *et al.,* 2008; Hughes *et al.,* 2009).

To measure lethal temperature (LTemp), insects are cooled or heated at a set rate, commonly $1^{o}C.min^{-1}$ (Hatherly *et al.,* 2004), or $0.5^{o}C.min^{-1}$ (Hughes *et al*., 2011), and then held at a predetermined temperature for a set time before being returned to the original temperature at the same rate of thermal change. For example, to assess the LTemp of the predatory mirid, *Nesidiocoris tenuis*, Hughes *et al.,* (2009) used a ramp of $0.5^{o}C.min^{-1}$ from rearing temperature ($23^{o}C$) to a range of set temperatures (between $-10^{o}C$ and $-15^{o}C$), insects were held at the set temperature for 15 minutes, after which they were raised back up to rearing temperature at $0.5^{o}C.min^{-1}$. After a recovery time the survival is assessed, before probit analysis is carried out to estimate the $LTemp_{50}$.

1.4.3. Rapid cold hardening

The ability of insects to rapidly (over minutes or hours) alter their cold tolerance is assessed through rapid cold hardening (RCH) experiments. Firstly, the discriminating temperature (DTemp) is determined by plunging the larvae from their culturing temperature (e.g. 20^{o}C), directly to a set sub-zero temperature (e.g. -10^{o}C) for a predetermined time (usually 2 hours). Similar samples are transferred to progressively lower temperatures for the same duration until the temperature at which roughly 80% mortality occurs is reached (up to 3% leeway either side is commonly reported). This temperature is defined as the DTemp. The ability of insects to RCH can then be assessed by acclimating larvae at various temperatures, e.g. 1h at 0^{o}C, before exposure to the DTemp. If the acclimated insects show increased survival, they are considered able to RCH. For example, the grain aphid, *Sitobion avenae*, had a DTemp of -8^{o}C when held for 3 hours, showing an 18% survival; but when acclimated at 0^{o}C for 2 hours before exposure to the DTemp, survival increased to 83% (Powell & Bale, 2004).

1.5. Mechanisms underpinning stress adaptations

Salt pioneered physiological studies on insect cryobiology despite only having relatively basic equipment. In the years succeeding, new technologies such as gas chromatography (Goto *et al.,* 2001), nuclear magnetic resonance (Overgaard *et al.,* 2007), electrophoresis (Zhang *et* al., 2012) and genetic analysis (Emerson & Bradshaw, 2010), have greatly expanded knowledge in the field, although it is still in its infancy regarding what mechanisms are involved in cold survival. It is clear that not all insects use all of the mechanisms discussed below, but generally the more mechanisms employed, or the greater the induction for any particular mechanism, the more cold hardy the insect becomes. These mechanisms can be part of diapause, but are not exclusive to the diapause programme. They can also contribute to enhanced cold tolerance through gradual acclimation (exposing the insect to a decreased temperature for several days), or RCH.

1.5.1. Cryoprotectants

Cryoprotectant literally means 'freezing protectant'. The term is commonly used in entomological cold tolerance studies, and refers to cold protective (rather than exclusively freezing protective) metabolites, i.e. it is used for both FA and FT species. Thus, these stress protective metabolites can act to prevent freezing, or to enhance non-freezing cold tolerance. The two most studied classes of cryoprotectants / stress protective metabolites are the polyols (polyhydric alcohols e.g. glycerol, sorbitol, mannitol, erythritol and inositol) and blood sugars (e.g. trehalose, erythrose, galactose, glucose and fructose). These substances are often produced both in response to, or in preparation for, low temperatures. Cryoprotectants usually share the same characteristics of being small, water soluble and stable molecules that are non-toxic even when accumulated to high levels. Compounds produced by insects act in either a colligative or non-colligative fashion. Colligative compounds, for example glucose, act in proportion to their concentration, and as such are usually accumulated to high concentrations. Non-colligative compounds, for example proline, interact with ice and prevent the growth and propagation of ice crystals, or prevent recrystallisation (preventing the expansion of ice in the haemolymph); they do not act in proportion to their concentration and therefore are often present at low levels (Marshall & Sinclair, 2012). Cryoprotectants are normally produced in the fat body and then released in to the haemolymph where they protect the insect from low temperature damage by decreasing the SCP or increasing osmolality in the cell.

The presence of glycerol and sorbitol in a diapausing silk moth, *Bombyx mori,* was first noted by Chino (1957). In many insects, glycerol is the most abundant polyol, and can make up 25% of the fresh weight of an insect (Tauber *et al.,* 1986). Glycerol is thought to act non-colliagatively as a stabilizer of proteins, increases repair of damaged proteins (Meng *et al.,* 2001) and stabilises membranes (Quinn, 1985). It is thought to be the most effective polyol due to its low molecular weight, which allows it to move readily through cell membranes. This allows it to decrease

osmotic stress inside the cell and limit cellular water loss; it also is highly soluble and has low toxicity (Salt, 1957). In the flesh fly, *Sarcophaga crassipalpis*, a slight elevation of glycerol was seen after an RCH response was initiated (Li & Denlinger, 2008). However, this response is not seen in all insects and a 25% decrease in glycerol was noted 2 hours after recovery, of RCH in the closely-related flesh fly, *S. bullata* (Teets *et al*., 2012). Earlier research by Yoder *et al*. (2006) on *S. bullata* suggested that glycerol was accumulated during low temperature stress, anoxia and desiccation; furthermore they showed that injecting glycerol led to a short term increase in cold tolerance and resistance to dehydration. Many of the polyols, including glycerol, are often derived from the catabolism of accumulated glycogen stores in the fat body (Worland *el al.,* 1998, Li *et al*., 2002). Glycogen catabolism is activated at temperatures between 5^{o}C and -5^{o}C by activation of glycogen phosphorylase by a signal transductdase cascade system (Pfister & Storey, 2002). Glycerol synthesis can be further enhanced by the diversion of carbon flow to the pentose phosphate pathway (Wood & Nordin, 1980).

The blood sugar trehalose is present in the blood at all times and is sometimes increased in the incidence of cold shock. Trehalose is thought to be able to stabilize membranes (Crowe *et al.*, 1992), which are commonly damaged during cold injury (Michaud & Denlinger, 2006). It is also present during heat stress, where it is thought to aid survival through stabilization of proteins (Bowler, 2005); this was also noted during freezing (Rudolph & Crowe, 1985). Trehalose is not always up-regulated in response to stress, for instance in *S. crassipalpis,* trehalose was significantly decreased after RCH (Michaud & Denlinger, 2007). It was suggested that trehalose may have been metabolised into glucose (and equivalents), to be used in the glycolytic pathway (Michaud & Denlinger, 2007). In the fall webworm, *Hyphantria cunea*, trehalose was the main cryoprotectant synthesised, though levels were higher in non-diapause pupae than diapause pupae; within the laboratory, acclimation to 5^{o}C or -5^{o}C significantly increased trehalose levels from 1.6mg.g^{-1} at 5^{o}C to 6.3mg.g^{-1} at -5^{o}C in non-diapause pupae. Field studies showed an increase in trehalose from January to March, after diapause has terminated (Li *et al*., 2001).

Trehalose has also been identified as an important cryoprotectant in desiccation and cross tolerance. When larvae of the Antarctic midge, *Belgica antarctica*, were exposed to 98% relative humidity (RH) until 50% of the body water was lost, trehalose concentrations increased from c. 6 to c. 19mg.g^{-1} of dry mass (Benoit *et al*., 2007). Although this rise could have been argued as passive due to the loss of body mass, when Benoit *et al.* (2009) injected trehalose in to *B. antarctica* resistance to both desiccation and thermal stress was enhanced. Larvae injected with 1.8µg of trehalose saw a significantly decreased water loss after 8 hours at 0% RH; while in a separate experiment, >70% of larvae survived exposure to -15^{o}C for 3 hours after being injected with 1.8µg of trehalose, non-injected controls (and Coast's solution injected controls) saw <35% of larvae survive the same treatment (Benoit *et al*., 2009).

1.5.2. Anti-freeze proteins

Anti-freeze proteins (AFPs) are able to lower the freezing temperature to below the melting temperature, a phenomena termed thermal hysteresis (Clark & Worland, 2008) and are synthesised in both FA and FT insects (fig. 1.4). This phenomenon is not isolated to insects, but is seen in fish (DeVries, 1971), plants, bacteria and fungi (Duman & Olsen, 1993). For instance, a typical fish AFP lowers the freezing point of fish plasma to -2.9^{o}C, roughly one degree below the freezing point of seawater (Duman & Devries, 1974). Over 50 insects are known to use AFPs (see Duman *et al*., 2004, for a full list), although many only show low levels of AFP production. AFPs act non-colliagatively and thus can be effective even at low levels (Kristiansen & Zachariassen, 2005). FA insects are able to lower the SCP through two primary roles, preventing inoculative freezing and impeding internal ice formation (Bale, 2002). In addition to the methods above FT insects benefit from ice nucleating agents (INAs) through prevention of recrystallisation, occurring, recrystallisation is the growth of an ice crystal, AFP's prevent the growth of ice crystals by covering the surface of the crystal, thus preventing liquid water getting in contact with ice, this decreases the nucleation temperature further preventing ice crystal formation

(Duman & Devries, 1972). Low levels of INAs in FT insects can also be beneficial, as recrystallisation will prevent damage whilst still allowing nucleation to occur, therefore preventing deep supercooling which leads to cell damage because of rapid ice formation (Tursman *et al.* 1994). The Coleopterid, *Cucujus clavipes puniceus,* has the highest recorded level of thermal hysteresis of 13^{o}C (Bennett *et al.,* 2005).

Insect AFPs have recently become the subject of much biotechnological interest, due to the discovery that some are more potent than fish AFPs. For example, the mealworm beetle, *Tenebrio molitor*, has a specific activity 100 times greater than a typical fish AFP (Graham *et al.,* 1997). This has led to the purification and sequencing of a number of insect AFPs (Graham & Davies, 2005; Graham *et al.,* 1997; Duman *et al.,* 1998) and the discovery that, like fish AFPs, there is very little similarity between sequences across species. This suggests that insect AFPs have evolved independently several times from different progenitors (Graham & Davies, 2005). One recent discovery relates to the use of AFPs in organ transplantation. When removed from a body, organs have to be held on ice to prevent degradation and can become damaged due to freezing; novel use of AFP in organ transplantation allows organs to survive cryopreservation. For example, fresh hearts of rats were transplanted after being preserved in fish AFP solution and showed significant transplant success, (Amir *et al*., 2005). As insect AFPs have a higher potency than fish AFPs, organs may be able to survive for longer than when fish AFP solutions are used.

1.5.3. Ice nucleators

The initiation of freezing often occurs at a nucleus; once ice has formed it promotes further organisation of water to form an ice crystal. This initiation is termed ‘ice nucleation’. There are two types of ice nucleation, homogeneous and heterogeneous (Denlinger & Lee, 2010). Homogeneous ice nucleation is due to electrostatic attraction between water molecules, combined with a decrease in thermal movement, whereas heterogeneous nucleation is

catalyzed by a substance other than water – an ice nucleating agent (INA) (Zachariassen & Kristiansen, 2000). Heterogeneous ice nucleation usually occurs at a higher temperature than homogeneous ice nucleation (Zachariassen & Kristiansen, 2000). INAs in FT insects were first identified in the darkling beetle, *Eleodes Blanchardi* (Zachariassen & Hammel, 1976) and have subsequently been seen to have an essential role in increasing the temperature of crystallization, which causes ice crystals to form in the extracellular spaces at higher sub-zero temperatures, (Knight, 1967). For example the New Zealand cockroach, *Celatoblatta quinquemaculata*, shows high levels of thermal hysteresis in gut tissues 1.99^{o}C and gut contents 0.56^{o}C, but no hysteresis in muscle, head or fat body cells (Wharton *et al*., 2009). When freezing occurs, osmotically active liquid water is lost in extracellular spaces, which decreases the water potential in extracellular spaces. This decrease in water potential drives water out of the cell (fig. 1.6; Bale, 1996), causing an increase in osmolality in the cell which, when combined with compatible solutes, protects the cell from intracellular ice formation (Duman *et al.*, 2004).

There are 5 main classes of INAs, their group and thermal activity range is shown in Table 1.2. INAs promote nucleation at relatively high temperatures compared to an insects SCP where no INA's are present, insects are known to be able to survive many tens of degrees below their SCP (Sinclair *et al.*, 2003b). FT species are also known to produce AFPs alongside INAs. This may be to restrict any size increase of ice crystals (recrystallisation), which would lead to physical damage of cells (Wharton *et al.,* 2009).

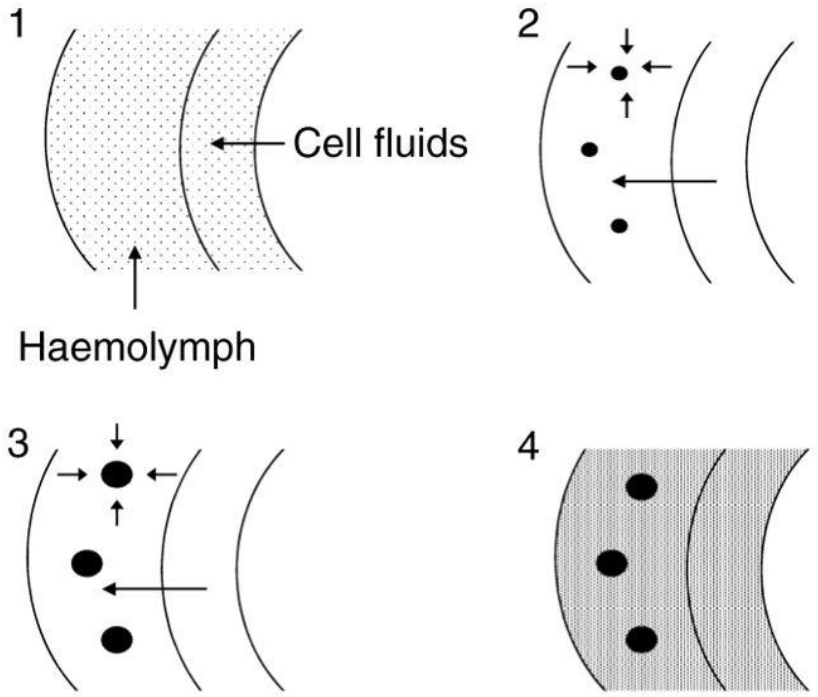


Figure 1.6. Action of ice nucleating agents in the formation of an ice crystal at sub-zero temperatures (Bale, 1996). 1. Equilibrium of haemolymph and cell fluids. 2. Initiation of freezing in haemolymph by INAs. 3. Growth of crystals drawing water from cells via an osmotic gradient. 4. Growth of ice crystal stops as haemolymph and cell fluids regain osmotic balance, both cellular fluids and haemolymph become concentrated, reducing the risk of harmful intracellular freezing. Figure taken from Bale (1996)

INAs are not universally produced by FT insects. For example, in overwintering adults of the freeze tolerant spruce bark beetle, *Ips typographus*, INAs were not found during winter months, adults instead relied on a high concentration of cryoprotectants which increased their $LTime_{50}$ at -10^{o}C from 2.5 to 13 weeks (Kostal *et al.,* 2011a). INAs are not seen in FA species, as these insects cannot survive any internal ice formation, and instead aim to evacuate all INAs prior to exposure to sub-zero temperatures. For example, non-diapausing larvae of the codling moth, *Cydia pomonella*, were noted as having low levels of ice nucleators in the haemolymph / gut, whereas diapause-destined larvae were noted evacuating all gut contents to remove potential INAs (Neven, 1999).

Table 1.2. Classes of ice nucleating agents and thermal range of activity of the agent. Table taken from Denlinger & Lee, (2010)

Type	Ice-Nucleating Activity	Cited
Ice nucleating proteins	-6 to -9^{o}C	Zachariassen & Hammel, 1976
Crystalloid compounds	-7 to -10^{o}C	Mugnano *et al.*, 1996
Ice nucleating bacteria	-2 to -10^{o}C	Kaneko *et al*., 1991; Lee *et a.,* 1991; Lee *et al*., 1993
Ice-nucleating fungi	-5^{o}C	Tsumuki *et al*., 1992
Inoculation by environmental ice	-1^{o}C or <MP of haemolymph, little or no supercooling	Tursman *et al*., 1994

1.5.4. Heat shock proteins

Heat shock proteins (HSPs) are a ubiquitous part of stress response from bacteria to humans (Feder & Hoffman, 1999). They are synthesised by organisms in response to stress factors including dehydration (Hayward *et al.* 2004), temperature extremes (Chen *et al.*, 2001b) or toxic substances (Yoshimi *et al.,* 2009). The role of HSPs in desiccation and heat stress is well studied, they have many functions within the cell including inhibition of protein aggregation (Fink, 1999), protein folding (Franck *et al.*, 2004) and disassembly (Bustard & Gupta, 1997), help modulate membrane fluidity (Tsvetkova *et al.*, 2002) and in plants, protein translocation across the cell wall and intracellular membranes (Bustard & Gupta, 1997; Hartl & Hayer-Hartl, 2002). HSPs are not just present in stressed cells, however, and play an important role in the regulation of protein folding in unaffected/unstressed cells. This was seen to be age dependent with older fruit flies, *Drosophila melanogaster,* producing an increased number of HSPs than younger flies (Fleming *et al.,* 1988). HSPs are highly conserved at a molecular level, this makes genetic analysis possible even if limited or no genome data is available for a particular species, as primers from other species can be used. Numerous families of HSPs have been identified, and these have been categorised according to their molecular weight in kiloDaltons. The most commonly studied heat shock proteins within insects are HSP90, HSP70 and small HSPs (sHSPs). HSP70 enhances stress survival by refolding damaged proteins, re-dissolving insoluble proteins, and tagging irreparable proteins for degradation; it was suspected that the full extent of its uses has not yet been discovered (Terlecky *et al.,* 1992), and still uses are being discovered (Voss *et*

al. 2012). Small heat shock proteins encompass a diverse range of proteins (12-40kDa), which all share the same alpha-crystalline domain (Shen *et al*., 2011). Their role in stress tolerance involves tagging early stage atypical proteins, inhibition of proteolysis (Vernon & Vanier, 2002) and refolding damaged proteins (for a review see Macarino *et al*., 1999). Small HSPs are known to be up-regulated in response to cold shock and heat shock within the flesh fly, *Sarcophaga crassipalpis* (Yocum *et al.,* 1998; Joplin *et al.,* 1990; Rinehart & Denlinger, 2000). HSP90 recognizes and repairs damaged protein that had been tagged by HSP70 and targets it for degradation (Young *et al.,* 2001). In some species, HSP levels can change as part of diapauses even in the absence of any stress. For example, in *S. crassipalpis* HSP70 and HSP23 levels are increased throughout diapause but levels of HSP90 were shown to decrease (Hayward *et al.,* 2005). The blow fly, *Lucilia sericata,* showed no change in the levels of HSP23, HSP70 or HSP90 during diapause in a non-stressed state (Tachibana *et al*., 2005). The expression of HSPs within diapause has been correlated with increased survival to cold (Rinehart *et al.,* 2000; Rinehart *et al.,* 2007). The expression of different HSPs is both species and life stage specific (see table 1.3). This may be due to the complex role of HSPs in both stress tolerance and regulating cell cycle arrest (Hayward *et al.,* 2005).

1.5.5. Membrane adaptations

Functioning cell membranes are essential for homeostasis and cellular physiology. There are three basic phase states of a membrane: a) highly ordered lamellar gel phase bilayer, b) a liquid crystalline fluid bilayer or c) a reversed hexagonal non-bilayer (Kostal, 2010). The alteration of a cell membrane from a liquid crystalline to a gel phase is of the most significant causes of cold injury under non-freezing conditions (Drobnis *et al*., 1993). Unregulated transition to the hexagonal phase usually results in cell death (Dowhan, 1997); though transition to the hexagonal phase is usually encountered due to desiccation rather than cold stress (Pruitt & Lu, 2008). To counteract this change, the membrane composition must be altered to increase

fluidity, preventing a gel phase occurring. By increasing the concentration of unsaturated fatty acids within membranes, the fluidity can be increased, and thus can help to keep the membrane crystalline at lower temperatures. It is perhaps not surprising, therefore, that membrane adaptations are closely associated with both diapause and cold tolerance.

Table 1.3. Regulation of HSP families for 13 species during insect diapause. 'Up' symbolizes an increase in HSP, 'down', a decrease in HSP and 'constant' no change in HSP levels. HSP were mostly analysed using mRNA rather than protein levels. Table is modified from MacRae, 2002.

Development stage	Insect	Molecular chaperone	Regulation
Larva	*S. nonagrioides* (corn stalk borer)	sHSP	Constant
		sHSP	Down
		Hsc70	Up
		Hsp70	Down
		Hsp90	Up
	O. fuscidentalis (bamboo borer)	Hsc70	Variable
		Hsp70	Down
		Hsp90	Down
Pupa	*S. crassipalpis* (flesh fly)	sHSP	Up
		sHSP	Up
		sHSP	Up
		sHSP	Up
		Hsp60	Up
		Hsp70	Up
		Hsp 70	Up
		Hsc70	Constant
		Hsp90	Down
	M. rotundata (solitary bee)	Hsc70	Constant
		Hsp70	Up
		Hsp90	Constant
Adult	*C. pipiens* (mosquito)	Hsc70	Down
		Hsp70	Constant
	D. triauraria (fruit fly)	sHSP	Constant
		sHSP	Constant
		Hsp70	Constant
		Hsp90	Constant

In altering the fluidity of the cell membrane, there are four changes that an insect can make:

1. Increasing the proportion of unsaturated fatty acids (UFAs) in comparison to saturated fatty acids (SFA) within a glycreophospholipid (GPL) membrane: Desaturation works due to a *cis* bond in the fatty acid chain being at 30^{o} to a saturated chain, this *cis* bond leads to the

molecule taking up more space, leading to a less efficiently packed bilayer, which decreases the temperature at which the transition from fluid to gel occurs (Wang *et al*., 1999). One such example of a change in the saturation of fatty acids was seen in the overwintering larvae of the rice stem borer, *Chilo suppressalis.* In non-diapause larvae, no more than 50% of fatty acids were unsaturated, whereas diapausing larvae from January (the coldest month recorded) observed a 30% increase in the percentage of unsaturated fatty acids (Izumi *et al.*, 2009). The desaturation of double bonds does not increase in all species, however, and in the flesh fly, *S. similis*, desaturation actually decreased as acclimation temperature decreased from 30 to 5^{o}C, though this decrease was not significant (Goto & Katagiri, 2011).

2. Decreasing the chain length of fatty acyl (FA): Due to their increased surface area for hydrophobic interactions, shorter fatty acyl chains have a lower melting point (Huang *et al*., 1997). This phenomenon is well researched in bacteria, with *Listeria monocytogenes*, reducing its average chain length from 15.6 to 15.3 at 10^{o}C (Zhu *et al*., 2005). This decrease in chain length was also noted in *Escherichia coli* in response to cold temperatures (Cooper *et al*., 1987). A decrease in fatty acyl chain length in insects is limited to just one study, which noted a decrease in chain length during chill tolerance of diapausing adults of the fire bug, *Pyrrhocoris apterus* (Tomcala *et al*., 2006).

3. Changing the ratio of GPL/cholesterol: In the gel phase, the conical shape of cholesterol disrupts the ordered membrane, increasing its fluidity. In a fluid membrane, an increase in cholesterol causes a highly ordered membrane; this decreases the permeability of the membrane and can disrupt membrane bound enzyme function (Haines, 2001).

4. Restructure head groups by increasing glycerophosphoethanolamines (GE) in proportion to glycerophosphocholines (GC). This was noted in the rice stem borer, *Chilo suppressalis,* which in summer conditions had a proportion of GE to GC of 1:1, whereas in winter, when diapausing larvae were exposed to low temperatures, this increased to 3:1 (Izumi *et al.*,

2009). The proportion of GE to GC does not change in all species. For example, the proportion of GE to GC does not change in *Chymomyza costata* during cold-acclimation whilst in diapause (Kostal *et al.*, 2003).

1.6. Desiccation stress

Roughly 70% of the body mass of most insects is made up of water. Without prior restructuring, organisms have a very limited tolerance to any variation in water volume, as it is known to disturb cellular function due to a change in solute concentration altering the interaction between proteins and enzymes (Holmstrup *et al*., 2010). During winter, most diapausing insects do not actively drink or take up water, as such, the loss of any internal water is a potential threat to survival. Desiccation stress is recorded in one of two ways, firstly, desiccation resistance is the measure of how quickly an insect loses water, secondly, desiccation tolerance can be observed, in which the amount (or percentage) of water that is lost before a defined amount of mortality is reached is recorded (usually 50% or just under 100%).

1.6.1. Mechanisms underpinning desiccation resistance

There are three primary routes for water loss in insects: cuticular, respiratory and defacation. The cuticle of an insect forms the waterproof barrier that protects the individual from desiccation stress. The thickness and composition of the cuticle are thought to alter evaporative water loss (Gibbs *et al.,* 1997), most overwintering insects reduce cuticular water loss to a minimum (Lundheim & Zachariassen, 1993). Instead the majority of water loss in diapause insects is thought to occurring through the respiratory spiracles during gas exchange (Zachariassen & Maloiy, 1989). For the same levels of gas exchange, insects are known to be more wasteful than other animals (Chown *et al*., 2011), this is not thought to be as a consequence of their relatively small size, but most insects have traded increased water loss so that they can purge CO_2 while preventing oxidative damage (Woods & Smith, 2010), or energy conservation (Matthews & White, 2011). During diapause, insects actively down-regulate

metabolism and therefore can increase desiccation resistance to water loss. In some species this reduction in metabolism may be as an active mechanism of water conservation (Chown & Davis, 2003). Desiccation resistance in *Drosophila melanogaster* has been studied, flies were selected for desiccation resistancs over 100 generations, by which time a marked difference in resistance was recorded (Gibbs *et al*., 1997). Desiccation resistant lines were shown to: a) have 30% more bulk water, with no increase in metabolically active water; b) lose 40% less water than control flies, of the water lost in both lines desiccation resistant strains lost 10% less water due to excretion; c) have no difference in cuticular hydrocarbon thickness. Although the direct mechanism for water loss was not directly found, results suggest that flies bred for desiccation resistance differed in either respiration or lipid composition (Gibbs, *et al.,* 1997), further research is needed to elucidate the full picture.

1.6.2. Mechanisms underpinning desiccation tolerance

1.6.2.1. Stress protective metabolites

As with cold tolerance, stress protective metabolites also play a key role in desiccation tolerance. Two particularly important metabolites within the desiccation response are trehalose and glycerol (Chown & Nicolson, 2004; Holmstrup *et al*., 2010). Trehalose is known to provide protection against the negative effects of cellular dehydration on protein and membrane integrity (Kikawada *et al*., 2007). Trehalose production in the Antarctic midge, *Belgica antarctica,* is proportional to the level of desiccation induced, desiccated larvae were then exposed to sub-zero temperatures, the increase in desiccation / trehalose was seen to confer an increase capacity to tolerate sub-zero temperatures (Benoit *et al*., 2009). In the same species, a metabolomics study (using GC-MS) of metabolites responsive to desiccation, identified increased levels of glycerol and erthritol (Michaud *et al.,* 2008). Freezing increased the same polyols in addition to mannitol, though whether the increase in these compounds was correlated with increased desiccation tolerance was not determined (Michaud *et al.,* 2008).

Both studies above suggest that there is a link between desiccation and cold tolerance mechanisms. An increase in the cryoprotectant glycerol was also seen in diapausing woolly bear caterpillars, *Pyrrharctica Isabella,* which had up to fivefold increase in glycerol concentrations when held in dry conditions (Layne & Kuharsky, 2000).

1.6.2.1. Aquaporins

Aquaporins are channels that water and small molecules (e.g. glycerol) can use to pass through cell membranes (Campbell *et al*., 2008). During dehydration and recovery, aquaporins provide an essential service in the rapid redistribution of water needed to achieve osmotic balance. In a recent study in the goldenrod gall fly, *Eurosta solidaginis,* survival of frozen fat body and mid-gut tissue was seen to be significantly reduced when aquaporin activity was blocked with an inhibitor (Philip *et al*., 2008). Aquaporin content was seen to alter with acclimation to desiccation and temperature (Philip *et al*., 2008). Increase in aquaporin content was also noted in the Antarctic midge, *B. antarctica*, in response to dehydration, rehydration and freezing (Yi *et al*., 2011).

1.6.2.2. Late Embryonic Abundant Proteins

Late embryonic abundant (LEA) proteins have recently been discovered to be associated with the desiccation tolerance of a range of organisms (Hand *et al*., 2011). So far, LEA proteins have only been recorded in a few insect species, including the chironomid, *Polypedilum vanderplanki,* (Kikawada *et al*., 2006), and they were shown to actively participate in the vitrification process by forming a glass state (Shimizu *et al*., 2010). In humans, LEAs are thought to prevent aggregations and maintain enzymatic activity in desiccation (Chakrabortee *et al*., 2007) and preserve membrane integrity in connection with sugars (Hand *et al*., 2011). However, research into LEAs in insects is still in its infancy.

1.6.2.3. Heat Shock Proteins

As with cold tolerance, HSPs appear to be important in the tolerance of desiccation. Benoit *et al*., (2010b) found HSP70 was up-regulated as a result of desiccation in three species of mosquito, *Ades asgypti, Anopheles gambiae* and *Culex pipens*. The study concluded that Hsp70 transcript abundance appeared not to alter water content or rates of water loss, but expression did lead to an increased tolerance of desiccation. An increase in HSP70 and HSP23 in dehydration, and HSP90 and HSP70 expression in rehydration was seen in non-diapause pupae of the flesh fly, *S. crassipalpis* (Hayward *et al*., 2004). More recently, in the African Chironomid *Polypedilum vanderplanki*, the expression of six heat shock protein genes (Pv-hsp90, Pv-hsp70, Pv-hsc70, Pv-hsp60, Pvhsp20 and Pv-p23) were noted as being up-regulated after 24 hours of desiccation <5%RH (Gusev *et al*., 2011). *Drosophila melanogaster,* do not appear to up-regulate HSP70 or in response to desiccation either at the protein or gene level (Goto *et al*., 1998; Sinclair *et al*., 2007 respectively), which suggests that different HSPs may be utilised in different species. HSP expression is a sign of damage being induced through severe desiccation / rehydration, thus its absence (particularly in a desiccation tolerant species) could infer a lack of damage, rather than a true lack of necessity to express the protein.

1.6.3. Cryoprotective dehydration

The term 'cryoprotective dehydration' was coined by Holmstrup & Westh (1994) after observing that supercooled cocoons of the earthworm, *Dendrobaena octaedra*, maintained at -3^{o}C, experienced water loss to ice. This was proposed to be because supercooled fluids have a vapor pressure higher than that of ice, and so water will be lost from the insect to the frozen external environment. This also occurs when insects are surrounded by frozen soil (fig. 1.7). Dehydration will continue until the water potential of the body fluid is the same as the water potential of the surrounding ice. It should be noted that the reverse of this is also true, i.e. if the water potential is lower inside the insect water will move from the surrounding so that an equal water potential

is maintained. Once the water potential of the insect has reached equilibrium, the risk of freezing is negated due to the melting point of body fluids equaling the ambient temperature (Denlinger & Lee, 2010). Cryoprotective dehydration has also been noted in the Arctic springtail, *Megaphorura arctica*, by cooling animals from either 5°C to -2°C or from 5°C to -6°C at a rate of 2°C per week (Thorne *et al.,* 2011). Through using gel electrophoresis and liquid chromatography tandem mass spectrometry, a diverse array of proteins involved in metabolism, membrane transport, cytoskeleton organization and heat shock and chaperone proteins were resolved (Thorne *et al*., 2011).

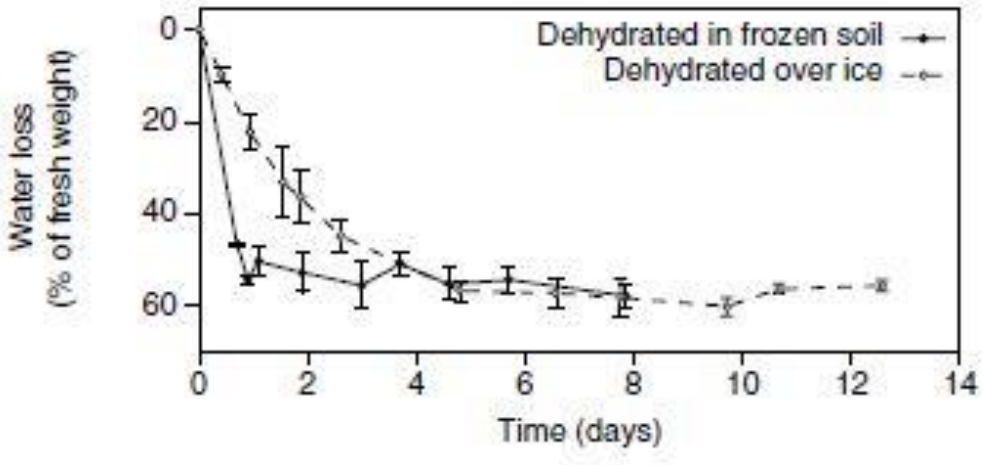


Figure 1.7. Water loss from supercooled earthworm cocoons (*Dendrobaena octaedra*). Cocoons were held in a small plastic cup on top of 5-7 ml of frozen soil/ crushed ice in a 14 ml glass vial at -3°C. The vial had a closely fitting lid to allow water vapor from the ice to saturate the air. The faster rate of dehydration in soil is thought to be due to the closer proximity of ice and the cocoon. Each point represents a mean of 4-6 observations (± SD). Figure taken from Holmstrup, 2002.

1.7. Metabolomics

Metabolomic techniques allow the characterisation of the unique chemical fingerprint of cellular processes; it has proved to be a powerful tool for the characterisation of the responses of organisms to a wide range of stressors. For example, dormancy (Andrews *et al.*, 2009; Bayley *et al.*, 2010), toxic insult (Bundy *et al.*, 2001; Ellis *et al.*, 2011; Poynton *et al.,* 2011) and disease (Kang *et al.,* 2011; Klein *et al.*, 2011). Metabolomics seeks to identify low metabolic weight substrates from the tissues or fluids of an organism; these include lipids, sugars, polyols and

amino acids, amongst other non-proteinaceous compounds. Due to metabolomics focusing on the substrate, it offers the most direct evidence of biochemical change within an organism. In many ways this is more powerful than observing gene, transcript or protein level changes, as it cannot be assured that the transcripts are translated and therefore have a direct effect upon the organism (Denlinger & Lee, 2007). Metabolites can be analysed using a range of technologies including gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR). Metabolomics has many advantages over older techniques (such as thin layer chromatography or high-performance liquid chromatography) as it offers simple sample preparation, high-throughput analysis (without chromatographic separation- ^{1}H-NMR), and high reproducibility. Also, in ^{1}H-NMR, all metabolites that contain a non-exchangeable H-atom are impartially assessed during one analysis rather than individual chosen compounds being assessed (Viant, 2008). A further advantage of metabolomics is in the provision of a powerful method for analysing non-model organisms, as no prior knowledge of gene or protein sequences is required, which is often a prerequisite in other 'omic' technologies.

In recent years, an increasing number of metabolomics studies on insect overwintering and cold hardiness have been completed, with a variety of stresses being researched, including rapid cold hardening (RCH) (Michaud & Denlinger, 2007; Overgaard *et al*., 2007; Teets *et al*., 2012), freeze tolerance (Michaud *et al*., 2008, Hawes *et al*., 2008; Kostal *et al.,* 2012), cold shock (Pedersen *et al*., 2008). To date, five studies have examined the effect of diapause on the metabolome of an insect (Michaud & Denlinger, 2007; Kostal *et al.,* 2011a; Colinet *et al*., 2012a; Xu *et al*., 2012; Zhang *et al*., 2012). Of the studies above, three were performed using NMR (Overgaard *et al*., 2007; Hawes *et al*., 2008; Pedersen *et al*., 2008) and the remaining were performed using CG-MS (Michaud & Denlinger, 2007; Michaud *et al*., 2008; Kostal *et al.,* 2011b; Colinet *et al*., 2012a; Kostal *et al.,* 2012; Xu *et al*., 2012; Zhang *et al*., 2012).

When assessing the two most up-regulated metabolites there appears to be links between several physiological responses. Glucose appears to be quite common being up-regulated due to desiccation (Michaud *et al*., 2008), cold tolerance (Overgaard *et al*., 2007; Michaud & Denlinger, 2007; Michaud *et al*., 2008) and diapause (Michaud & Denlinger, 2007). Proline is up-regulated in RCH (Teets *et al.,* 2012) and in diapause (Kostal *et al.,* 2012). Trehalose is up-regulated in diapause (Michaud & Denlinger, 2007; Xu *et al*., 2012), RCH (Michaud & Denlinger, 2007; Michaud *et al*., 2008) and cold shock (Michaud *et al*., 2008). As such, there appear to be links between different physiological states.

Metabolomics enables the closest link to how a stress affects a cell by observing the substrates and by-products of enzymatic reactions, this is the major benefit over older techniques as it allows the metabolome to be simultaneously assessed and a suite of metabolites can be readily identified, which has obvious advantages over older techniques in which the specific metabolites are targeted. As a large amount of data is normally generated an insight not only in to specific metabolites but on whole pathways can be generated, for example Michaud & Denlinger (2007) were able to identify the down-regulation of the Krebs cycle in *S. crassipalpis.* Metabolomics also has benefits for when the metabolites involved in the response are unknown, as not only are metabolites that are 'expected' assessed but those which are 'unexpected' are assessed too.

1.8. Climate change

As outlined in previous sections, insects have evolved a great diversity of strategies to cope with seasonal change, and in particular the cold stress associated with winter. Key to this success is the overwintering dormancy (diapause). However, there is increasing evidence that the programming of diapauses, and winter survival, may be altered by predicted climate change.

1.8.1. Current predictions

Global mean surface temperatures have risen by 0.74°C ±0.18°C over the last 100 years (1906-2005), with the rate of warming in the last 50 years double that of the last 100 years (0.13°C± 0.003°C vs. 0.07°C± 0.02°C per decade) (IPCC, 2007). This trend is not linear; with the period from 2001-2005 showing the greatest rate of change. It is predicted that by the year 2100, the average surface temperature could be on average up to 5°C higher, according to the A1Fl prediction (fig. 1.8).

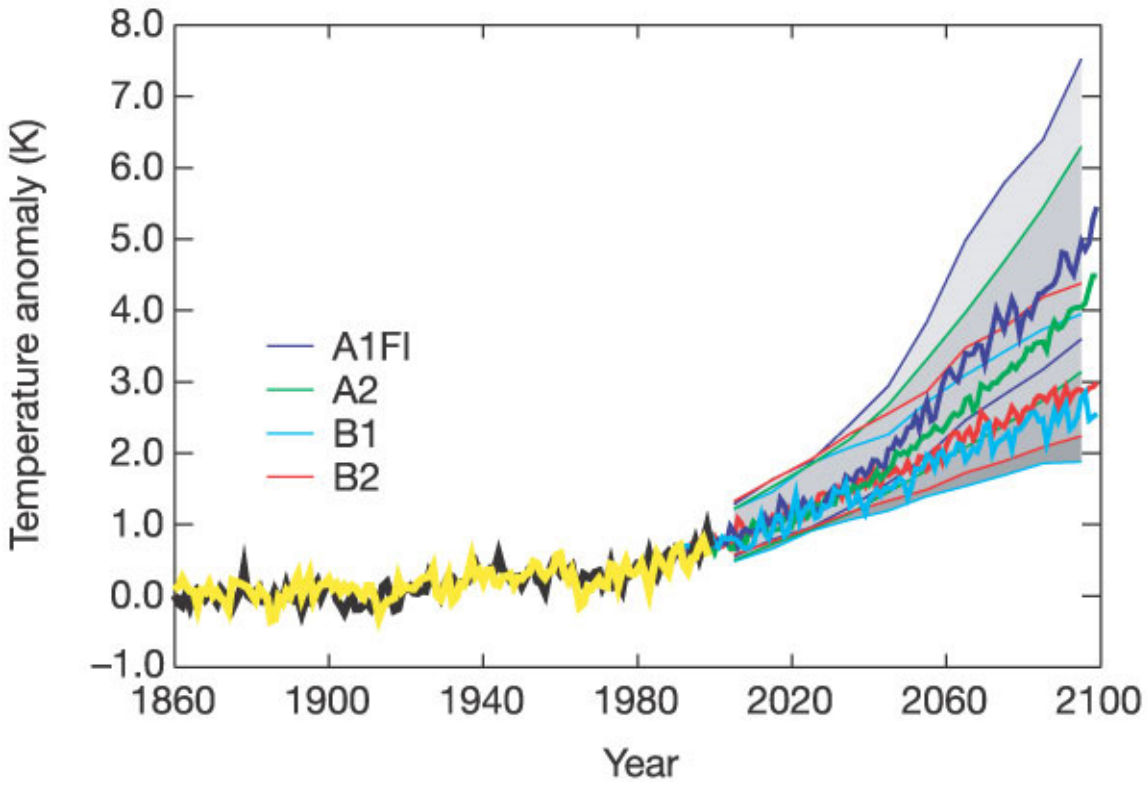


Figure 1.8. Predicted global-mean surface temperatures. Shown are predictions according to the SRES A1FI (blue), A2 (green), B1 (light blue) and B2 (red) scenarios, with their uncertainties (5 to 95 percentiles) shown as grey regions bounded by lines of the appropriate colour. Also shown are the observed temperatures in black, and a HadCM3 simulation including both anthropogenic (greenhouse gases, sulphate aerosols and tropospheric and stratospheric ozone) and natural (solar and volcanic) forcings in yellow. Figure taken from Stott & Kettleborough, (2002).

Although global temperature is likely to increase by up to 5°C by 2100, this change in temperature is not equally distributed, with the greatest amount of warming likely to be in the Arctic and sub-Arctic regions, this is known as Arctic amplification (MacDonald, 2010), though some areas of the Antarctic will also show large increases. A review of temperature data from

1958 to 2008 showed that, while most areas of the world showed a modest rise in temperature, a limited number of areas of Antarctica saw a decrease in temperature of up to 4°C, whereas the majority of the Arctic and sub-Arctic saw a temperature rise of between 0.5 and 4°C (fig. 1.9). If this model of Arctic amplification extends to 2100, a global increase of 5°C could result in a significantly larger change in temperature within the U.K. and at more Northerly latitudes. Models of climate change also predict an increase in the number of extreme weather events, especially temperature extremes (Easterling *et al*., 2000). Thus, while spring, summer and autumn conditions may get warmer, winter cold will still be a survival threat – as evidenced by the recent coldest winters on record in northern Europe. As insects are poikilotherms, this could have dramatic effects on insect phenology, distribution and abundance.

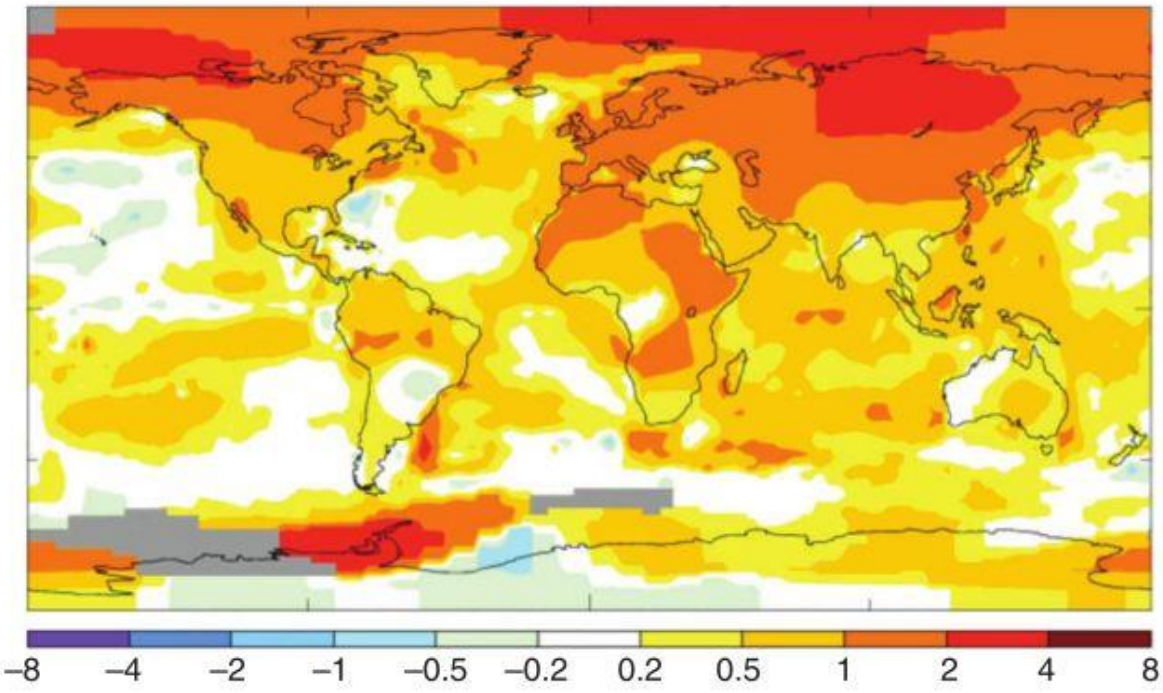


Figure 1.9. Global temperature trend from 1958 to 2008 (data and mapping algorithm from NASA Goddard Institute for Space Studies). Figure taken from MacDonald, (2010).

1.8.2. The impact of climate change on insect phenology, distribution and abundance

From figure 1.9, it can clearly be seen that insects from temperate, boreal, sub-Arctic and Arctic/ Antarctic zones will experience the largest temperature increase. Consequently, species in these regions will be the focus of this section. During spring and summer, climate warming is likely to have few detrimental effects on most insect species. As temperatures increase, rates of development will increase, which may allow an increased number of generations per year. For example, populations of the codling moth, *Cydia pomonella*, in Switzerland are currently mostly univoltine with a 20% chance of a second generation (Stoeckli *et al.,* 2012). Under predicted climate change, however, it is predicted that this species will become tri-voltine (Stoeckli *et al*., 2012). During autumn and winter, many species in temperate and poleward zones enter diapause, and there are emerging concerns over the loss of synchrony between diapause controlling factors, photoperiod and temperature under climate change (Bale & Hayward, 2010). While temperatures are predicted to increase with climate change, particularly in autumn, photoperiod will not. Most insects need to be cued by CDL and appropriate temperatures to enter diapause. For instance, if held above 12-15^{o}C (dependent on geographic location of strain), larvae of the blow fly, *Calliphora vicina* will terminate diapause regardless of photoperiod experienced by the adult generation (Vinogradova & Zinovjev, 1972). Some species may be able to adapt rapidly to climate change, e.g. field studies in the pitcher plant mosquito, *Wyeomia smithii*, have shown that adults can alter their CDL to stay synchronised with the increase in ambient temperatures from 1972 to 1996 (Bradshaw & Holzapfel, 2001). This shift in CDL was also noted in the fall webworm, *Hyphantria cunea* (Gomi *et al*., 2007).

One of the biggest concerns for overwintering insects is nutrient depletion, as once an insect is in diapause it often will completely cease feeding until diapause is terminated. As temperature increases, the metabolic rate within diapause also increases for many (though not all) species. If insects have a higher metabolic rate, they will use up energy stores quicker. For example, the

freeze-tolerant goldenrod gall fly, *Eurosta solidanginis*, overwinter either in goldenrod stems above the snow layer (exposed) or in broken stems at ground level (Irwin & Lee, 2003). Snow covers the ground level stems, and acts as a thermal buffer, which means that these larvae are more metabolically active as they remain unfrozen and use more energy overwinter than their above snow level counterparts. Spring emergence was seen to be significantly lower in ground level larvae, compared to those exposed to colder above ground temperatures (83.5% vs. 93.0%) (Irwin & Lee, 2003). The effects of snow cover on the energy usage have also been investigated in the Arctiid caterpillar, *Pyrrharctica Isabella* (Marshall & Sinclair, 2012b). These authors identified a 38% increase in lipid consumption in those caterpillars that overwintered below snow level, though larvae held above snow level did have a higher survival rate (57% vs. 45%) (Marshall & Sinclair, 2012b). Where climate warming does occur, the amount of snow cover is likely to decrease in many locations, and the impact of this on insect overwintering success requires further investigation.

Where survival is affected by winter conditions, species distribution is also likely to change as global warming continues. For example, in southern green stink bug, *Nezara viridula*, the northern boundary is defined by the minimum temperature experienced during winter (Kiritani et al., 1966; Kiritani, 1971). Due to climate change, temperatures in Osaka, Japan have risen by approximately 2^{o}C from 1950 to 2000; from 1960 to 2000, *N. viridula* range expanded from 34.2^{o}N to 34.7^{o}N (Kunzig, 2005). By 2008 their range had further expanded to 34.8^{o}N (Takeda *et al.,* 2010). There is also some evidence that the southern boundary of some species may be moved northward in future years, for example, Kellerman *et al*., (2012) assessed the upper thermal limits of 94 *Drosophila* species, which were found to be less plastic than lower thermal limits, due to low evidence for spatial associations driving common adaptations. This was supported by Addo-Bediako *et al*. (2000) who found that there is low variance in maximal heat resistance, with low evolutionary potential for raising the thermal limit (Mitchell & Hoffmann,

2000). Due to this, it was concluded that many of the species that were already close to their upper thermal limit would be at risk from climate change.

1.8.3. Broader impacts of disrupted insect phenology

Insects have a wide range of roles in our ecosystems: they pollinate plants, are used in biocontrol and are the vectors of disease (both plant and animal). The synchrony between plants and insects is particularly important for pollination, as some plants require specific species to provide a pollination service. If the synchrony of flowering and insect presence is mismatched, flowers cannot successfully reproduce. One such example is the glacier lily, *Erythromnium grandiflorum,* which flowers just after snowmelt in spring, and are pollinated by bumble-bee queens of *Bombus bifarius* and *Bombus occidentalis* (Thomson, 2010). Thomson (2010) noted a growing desynchronisation, with *E. grandiflorum* flowering earlier, before bees had emerged, which led to a decrease in fruiting success. Although insects are essential for pollination in agriculture, they are known to damage crops in three ways, herbivory through defoliation (e.g. locusts), herbivory through removing phloem (e.g. aphids) or transmission of diseases e.g. *wheat dwarf virus* (WDV) whose vector is the leafhopper, *Psammotettix alienus*. In Sweden, WDV is most serious when transmitted to winter wheat during autumn, as by spring the mature plant is resistant to the disease (Lindblad & Sigvald, 2004). When autumn temperatures are below 10^{o}C, leafhoppers are not as active and transmission of WDV is low; when field temperatures are above 10^{o}C, WDV transmission is high (Lindblad & Areno, 2002). As temperature increases, the number of days in autumn >10^{o}C will increase, which will offer farmers the choice of either planting at the same time and spraying insecticide if temperatures are warmer, or planting crops later when temperature (and photoperiod) has decreased. Both options may lead to a lower net income. The main effect that climate change will have on agriculture is an increase in the temporal abundance of pest species; this is particularly true of aphids which are known to carry a wide variety of diseases and have multiple generations each

year (Bale & Hayward, 2010). Mild winters and warm springs allow early populations of aphids to produce winged migrants, which migrate to germinating crops causing potentially catastrophic losses, whereas severe winter weather causes higher mortality and winged migrants are produced once crops are hardened off, reducing the risk of severe damage (Bale & Hayward, 2010). The impact that climate change has on the insect transmission of human diseases, such as malaria is not yet fully understood (Randolph, 2009). This is due to the complication of political, cultural, biological and environmental factors; this has led to the conclusion that over recent decades there has been no infectious disease that has been spread through the effects of global warming alone (Randolph, 2009). There are, however, incidences where an increase in tick-borne encephalitis (TBE) has been increased when spring temperatures were high (Lindgren & Gustafson, 2001), conversely TBE was increased in years where spring temperature was low (Randolph, 2000) suggesting other factors may be involved.

1.9. Choice of study system

The insect chosen for this study was the blow fly for the following reasons:

To avoid the risk of erroneous results due to the use of inbred insects, field fresh populations were to be established; as such the species used must be native to the U.K. and common enough for a large number of wild insects to be collected to ensure a representative sample size.

Calliphora vicina is a widespread and common species in the U.K. (Erzinclioglu, 1996). It has a distinct diapause phenotype (Saunders, 1987) that is closely associated with an increase in cold tolerance (Saunders & Hayward, 1998). *Calliphora vicina* also has sensitivity to high temperature in respect to terminating diapause; Vinogradova (1974) found that larvae would terminate diapause (termed 'reactivation' in her research) if exposed to temperatures between 12^{o}C and 17^{o}C, depending on their geographic location. U.K. strains appear sensitive to temperatures

above 15°C as both adults and L3 larvae reduce incidence of or terminate diapause (Saunders, 1987; Saunders & Hayward, 1998). For these reasons, this species was selected for this study on insect diapause, stress adaptation and the impact of climate change on diapause and insect phenology. *Calliphora vomitoria* was selected as a comparator species, to determine whether strategies were similar between evolutionarily closely-related species (based on mitochondrial cytochrome c oxidase subunit I (COI) analysis, Boehme *et al*., 2012), but with distinct differences in habitat preference.

1.9.1. Calliphora vicina

The blow fly, *Calliphora vicina* Robineau-Desvoidy (Diptera: Calliphoridae) is widely distributed throughout Europe (Fischer *et al.,* 2004; Hwang & Turner, 2005), North America (Slone & Grunder, 2007), Africa (Williams & Villet, 2006), Asia (Liu *et al*., 2011), Australia (Stevens & Wall, 2001), New Zealand (Howlet, 2011) and is present in both the sub-Arctic (Aak *et al*., 2011a, b) and sub-Antarctic (Lebouvier *et al*., 2011). It is freeze intolerant (Block *et al*., 1990), and as such it needs to avoid the stressful sub-zero temperatures seen during winter in these areas. *Calliphora vicina* achieves this through physiological mechanisms (e.g. diapause) or behavioural choices such as seeking warmer sites to overwinter. *Calliphora vicina* is synanthropic (lives near and benefits from the artificial habitats that humans create) and is often seen flying indoors, though it has a wide distribution inhabiting both rural and urban areas. Due to their synanthropic nature, Erzinclioglu (1996) suggested that *C. vicina,* particularly in Southern Britain, did not need to enter diapause for winter survival. For populations residing further north, diapause is essential for winter survival (Vinogradova, 1986). In the U.K., larvae enter a facultative diapause, with *C. vicina* at 51°N being thought to have four generations per year (Graham-Smith, 1916). At the northern boundary of *C. vicina* in Russia it is thought to have an obligate diapause, having just one generation per year (Vinogradova, 1975; Vinogradova, 1986). In the U.K., however, *C. vicina* has a short life cycle (fig. 1.10) taking just 32 days to develop from

egg to egg at 15°C (Davies, 2006). It enters diapause as a post-fed 3rd instar larva (Richard & Saunders, 1987; Saunders, 1987). The Russian strain of *C. vicina* is thought to enter an adult diapause in addition to a larval diapause (Vinogradova, 1986).

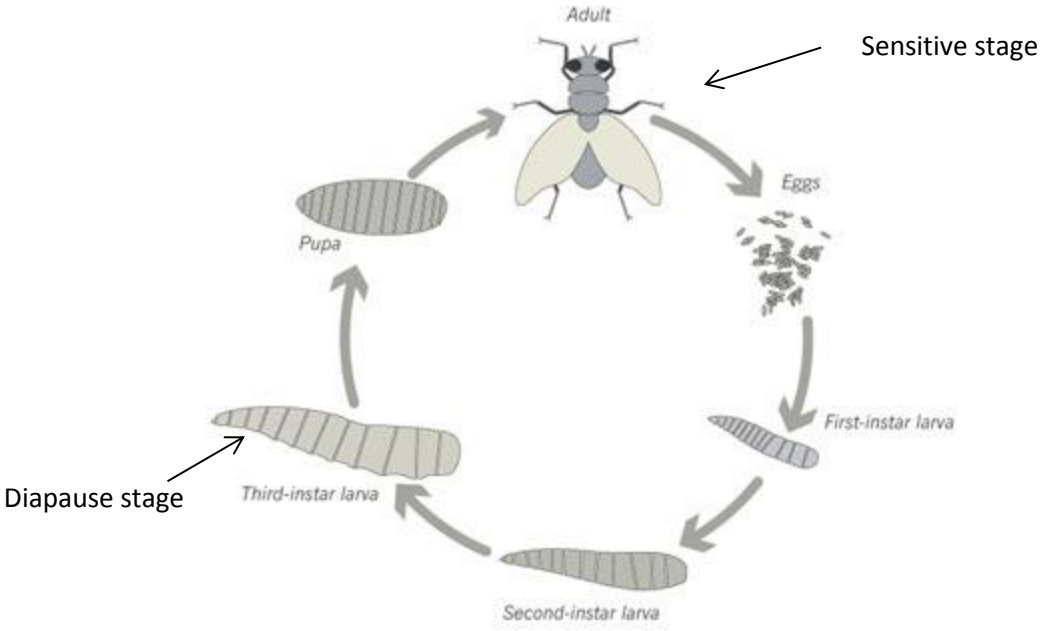


Figure 1.10. Life cycle of *Calliphora vicina*. Adults lay eggs upon meat, which hatch and progress through 1st (L1), 2nd (L2) and 3rd (L3) instar stages. During the L3 stage, larvae stop actively feeding and move away from the meat (termed wandering). During this stage, larvae will either remain as larvae and enter diapause, or pupate and continue development to adults. Diapause incidence is determined by adults (sensitive stage) experiencing the critical day length (CDL), together with appropriate temperature cues.

Diapause in *C. vicina* is induced in adults of the maternal generation and occurs in third-instar (L3) larval progeny (Vinogradova and Zinovjev, 1972; McWatters & Saunders, 1996). Diapause induction requires the correct environmental cues to be 'sensed' in the maternal generation before diapause can be triggered. As with the majority of temperate insects, the two main environmental factors controlling diapause in *C. vicina* are photoperiod and temperature. The CDL is dependent on latitude, for instance the CDL for a southern English strain of *C. vicina* is 14.5 hours of light, whereas the more northerly Finnish strain requires 16 hours to induce diapause in the next generation (McWatters & Saunders, 1998). Temperature has also been shown to be important in the decision to enter diapause. If diapause induced larvae of *C. vicina*

are held at a constant temperature above 12 to 17^{o}C (dependent on geographic source of the strain), larvae will avert diapause and pupate (Vinogradova & Zinovjev, 1972). Chernysh *et al.,* (1995) found that, between the egg and wandering stages, temperature had no effect on the decision to enter diapause, although larvae were very sensitive to temperature whilst wandering. Diapause aversion due to high temperature can be an advantage during mild weather, as another generation may be completed before the temperature gets too cold for development to progress. Conversely, if the temperature drops too quickly, development may slow too much, preventing the insect from completing another life cycle to reach the 3rd instar stage that is required to enter diapause. Thus, the insect could succumb to winter cold and this may be a problem with respect to climate change.

Through taking eggs, larvae, pupae and adults of *C. vicina* down to their SCP Block *et al*. (1990), it was found that freezing at all life stages was lethal, as such *C. vicina* is a freeze intolerant species. The SCP of non-diapause eggs was the lowest at c. -25^{o}C, the SCP of L1 through to adults ranged from c. -14^{o}C to -9^{o}C. The SCP of diapause life stages has never been measured. Cold tolerance at 0, -4 and -8^{o}C in three strains (Finland, 65^{o}N, Scotland 55^{o}N and Italy 44^{o}N) of *C. vicina* was assessed by Saunders & Hayward (1998), and D larvae were found to be more cold tolerant than ND larvae. Also, at 0^{o}C, larvae from higher latitudes were seen to be more cold tolerant than those at lower latitudes. *Calliphora vicina* were concluded as being moderately chill tolerant (Saunders & Hayward, 1998).

The concentration of a number of cryoprotectants present in ND *C. vicina* at four life stages (egg, L3, pupae and adults) were Investigated by Block *et al*., (1990) (table 1.4.). Eggs were surprisingly devoid of polyols and sugars (cryoprotectants), with only glucose being present in measurable quantities. The greatest concentration and number of quantifiable cryoprotectants was greatest in ND L3 larvae which saw glucose fructose, mannitol, sorbitol and myo-inositol present. The cryoprotectants present in diapause larvae have not been investigated to date.

Table 1.4. Cryoprotectants identified in four life stages of *C. vicina*. Mean ±SD concentrations are given if >1mg.g^{-1}; T symbolizes 0.1-1 mg.g^{-1}; + symbolizes<0.1 mg.g^{-1}; - symbolizes cryoprotectant not detected. Table taken from Block *et al.*, (1990).

Compound	Eggs n=5	L3 n=7	Pupae n=4	Adults n=9
Glucose	1.26 ± 0.52	5.80 ± 0.95	3.36 ± 2.49	8.22 ± 6.50
Fructose	T	1.52 ± 0.97	T	3.29 ± 2.40
Sucrose	+	T	T	T
Trehalose	+	T	2.09 ± 0.94	T
Glycerol	T	T	T	T
Mannitol	-	1.88 ± 0.84	T	+
Ribitol	T	T	T	T
Sorbitol	-	2.32 ± 0.33	1.15 ± 1.15	T
Xylitol	-	-	-	T
Myo-inositol	T	1.62 ± 0/73	T	+

The primary ecological function of flesh flies, such as *Calliphora vicina* is to facilitate the decomposition of dead animals, i.e. nutrient cycling (Aak *et al.,* 2011a). *Calliphora vicina* is also known to be an effective pollinator, for example of the hybrid carrot, *Daucus carota* (Howlett, 2011), onion, *Allium cepa* (Currah & Ockendon ,1984), turnip, *Brassica rapa*, (Rader *et al.,* 2009) and alfalfa, *Medicago citrana,* (Perez-Banon *et al*., 2003). Supplementation of agricultural crops with *C. vicina* to aid pollination does not currently occur, but as *C. vicina* are native to the U.K., as well as cheap and easy to culture, there would seem scope for this to change. Though it should be pointed out that *C. vicina* are known to carry diseases (Vinogradova, 2009), as such the site of release should be carefully considered. *Calliphora vicina* is also a key species of interest to forensic science. *Calliphora vicina* are known to rapidly colonize a corpse (Reibe & Madea, 2010). Due to well-studied temperature dependent development (Ames & Turner, 2003; Kaneshrajah & Turner, 2004; Donovan *et al*., 2006; Niederegger *et al*., 2010), *C. vicina* can be used to very accurately age a corpse to give a post mortem interval, which is often more accurate than an autopsy (Ament *et al*., 2004). An example of development rates of *C. vicina* at three temperatures is given in table 1.5. This detailed understanding of day degree requirements could also help in the development of detailed phenology models.

Table 1.5. Development rates of multiple life stages of *C. vicina* over a range of temperatures. Table taken from Anderson, (2000).

	15.8 ± 0.004°C Time to reach stage (h)		20.6 ± 0.03°C Time to reach stage (h)		23.3 ± 0.02°C Time to reach stage (h)	
Stage	Min	Max	Min	Max	Min	Max
1st instar	41.4±1.2	46.7±0.6	22.5±0.2	36.0±4.0	21±0	29.5±5.2
2nd instar	83.0±10.0	88.3±12.7	57.0±6.0	57.0±6.0	45.0±0	52.0±0
3rd instar (feeding stage)	128.0±9.0	146.0±15.6	84.0±10.0	93.5±0.5	77.0±0	85.0±8.0
Prepupal	228.0±3.3	257.0±9.6	155.5±4.2	162.5±1.1	146.0±0	173.0±0
Pupal stage	294.0±4.7	440.3±42.1	213.0±4.5	233.0±0.9	202.8±5.8	279.0±22.5
Adult	719.7±6.0	874.6±20.7	514.8±3.7	572.0±10.0	454.0±6.0	499.5±7.5

1.9.2. Calliphora vomitoria

Calliphora vomitoria is *C. vicina's* closest relative, based on COI data (Whitworth *et al.,* 2007). *Calliphora vomitoria* appears to have a wide distribution in the Northern hemisphere, being noted in Britain (51.3°N, Hwang and Turner, 2005; 54.5°N, Davies, 1999), USA (37.4°N, Brundage *et al*., 2010), Germany (50.9°N, Niederegger *et al*., 2010; 60°N, Reibe & Madea, 2010), France (Bourel *et al.,* 1999), Ankara, Turkey (39.8°N; Sabanoglu & Sert, 2010), Spain (43°N; Baz *et al*., 2007), Poland (52.3°N, Matuszewski *et al*., 2008), Huanslong, China (32-33.1°N, Zheng *et al*., 2011), Urmia, Iran (37.3°N, Tűzűn *et al*., 2010) and Hokkaido, Japan (43.5°N, Iwasa & Inoue, 2012). *Calliphora vomitoria* does not have the strong synanthropic tendencies of *C. vicina*, being found to be more abundant in rural areas (Hwang & Turner, 2005; Pohjoismaki *et al*., 2010). *Calliphra vomitoria* is widely distributed throughout England, where the ranges of *C. vomitoria* and *C. vicina* overlap, *C. vicina* usually dominate in abundance (Pohjoismaki *et al*., 2010). *Calliphora vomitoria* predominantly colonise larger carcasses (Henning, 1950; Davies, 1990; 1999), with smaller carcasses, such as mice, attracting no *C. vomitoria* adults (Davies, 1990). The temporal abundance data for *C. vomitoria* suggests that they are less abundant year round than *C. vicina* and were completely absent from September to May, whereas *C. vicina* is present until December and begins to be found again in April (fig. 1.11) (Hwang & Turner, 2005). However, it should be noted that this study was conducted in London and *C. vomitoria* is noted to be a more

rural species. *C. vomitoria* also appear to prefer higher altitude habitats, for example in Spain they dominate at 1500-1900m (79.5% of adults captured), whereas the blow fly *Chrysomya albiceps,* dominated at lower altitudes <1200m (Baz *et al*., 2007). This may be due to temperature, as in Hokkaido, in summer *C. vomitoria* saw their highest abundance in highland areas (1000-1150m), but in autumn abundance peaked at lower altitudes (70 - 500m) (Iwasa *et al*., 2012).

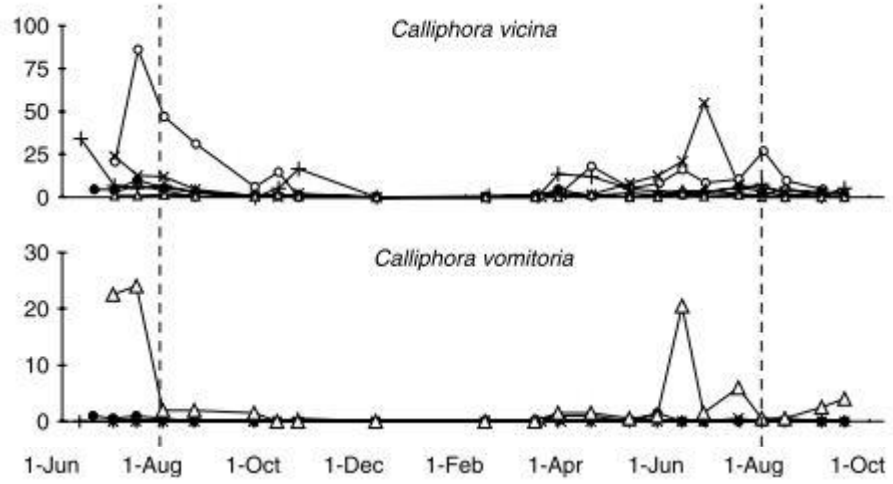


Figure 1.11 Temporal abundance of *Calliphora vicina* and *Calliphora vomitoria* from June 2001 to October 2002. Vertical axis shows the number caught per day. Taken from Hwang & Turner (2005).

The life cycle of *C. vomitoria* is thought to be identical to *C. vicina* (fig. 1.10). The only difference may be the stage at which diapause is induced and the stage at which diapause is carried out. There have been reports of *C. vomitoria* successfully entering diapause in both L3 and adult stages (Vinogradova, 1986). However, to our knowledge, the conditions under which diapause is induced have not yet been characterised. There are also suggestions that *C. vomitoria* does not enter diapause (Fraser, 1959; Block *et al.,* 1988) suggested that *C. vomitoria* overwinter as non-diapause larvae in the soil.

Calliphora vomitoria is thought to be freeze intolerant as it was unable to survive exposure to its SCP at any life stage (Block *et al*., 1990). During this study, the SCP of all life stages was assessed and found to be comparable with those of *C. vicina,* with eggs having the lowest SCP at c. -24^{o}C,

and the SCP of L1 through to adults ranging from c. -11°C to -7°C. The presence of cryoprotectants in four life stages was also analysed using gas chromatography (Table 1.6). In general a fewer cryoprotectants were in measurable quantities compared to *C. vicina*. Adults had the highest number of quantifiable cryoprotectants, with glucose and fructose being the most abundant.

Table 1.6. Cryoprotectants identified in four life stages of *C. vomitoria*. Mean ±SD concentrations are given if >1 $mg.g^{-1}$; T symbolizes 0.1-1 $mg.g^{-1}$; + symbolizes<0.1 $mg.g^{-1}$; - symbolizes a cryoprotectant that was not detected. Table taken from Block *et al.*, (1990)

Compound	Eggs n=6	L3 n=6	Pupae n=6	Adults n=6
Glucose	T	T	T	4.50 ± 1.75
Fructose	T	T	1.02 ± 0.34	3.08 ± 1.69
Sucrose	-	T	T	T
Trehalose	-	-	T	-
Glycerol	T	1.49 ± 0.35	T	1.47 ± 0.68
Mannitol	-	T	T	T
Ribitol	+	T	T	T
Sorbitol	-	-	T	T
Xylitol	-	-	-	-
Myo-inositol	T	1.02 ± 0.77	T	+

As was noted in *C. vicina, C. vomitoria* is a known pollinator (though not of agricultural crops) and has uses in forensic science. *Calliphora vomitoria* is the primary pollinator of the slipper orchid, *Cypripedium flavum* (Zheng *et al*., 2011). As was described for *C. vicina*, the development of *C. vomitoria* on human cadavers can be used to calculate an accurate PMI (Ames & Turner, 2003).

1.10. Objectives of the study

Using *C. vicina* as the main study species, the core objectives of this project were to:

1. Evaluate how *C. vicina* responded to cold and desiccation stress and observe differences in cold tolerance within and outside the diapause programme.
2. Assess the impact of predicted climate change on the diapause phenotype of a common (and important) U.K. insect species. This includes the effects on overwintering survival and seasonal phenology.
3. Characterise the metabolic fingerprint of D and ND developmental stages, and identify putative metabolites underpinning the enhanced stress tolerance phenotype and developmental arrest associated with diapause.
4. Compare and contrast the cold and desiccation stress physiology of *C. vicina* with its most closely-related species, *C. vomitoria.*

2. Differences in culturing, diapause induction and initiation between two closely-related Calliphoridae species and their cross.

2.1. Summary

A comparison of the culturing of diapause and non-diapause *Calliphora vicina* and *C. vomitoria* was conducted. *Calliphora vomitoria* responded well to culturing techniques used on *C. vicina*, although the following differences were noted: a) The time required for *C. vomitoria* to develop from eggs to wandering L3 larvae increased by 10.2 days. b) In *C. vicina* it has long been established that diapause is maternally regulated, with adults exposed to a long day (LD) photoperiod bearing non-diapause larvae, while adults exposed to a short day (SD) photoperiod bear larvae which are able to enter diapause. This study supports this current understanding with 14.4% of LD *C. vicina* entering diapause and 77.2% of SD entering diapause. In *C. vomitoria,* just 9.3% of insects entered diapause under SD conditions, and just 2.7% under LD conditions. Concluding that *C. vomitoria* do not enter diapause under conditions administered in this study. c) *Calliphora vomitoria* held in dry sawdust had a high mortality rate (53.4 $\pm$ 6.0%), which was reduced to 3.4 $\pm$ 1.2% when larvae were held in a medium containing an equal weight of water to sawdust.

Interestingly, female *C. vomitoria* and male *C. vicina* were able to produce an F1 hybrid. A hybrid was not possible for the opposite cross. Egg viability in the female *C. vomitoria* and male *C. vicina* hybrid was low, at just 16.7%. The F1 hybrid were able to successfully produce an F2 generation. Both the F1 and F2 generation were seen to have *C. vomitoria* like morphology.

This research furthers our understanding of the biology of both *C. vicina* and *C. vomitoria* and provides better methodology for the culturing of *C. vomitoria*.

2.2. Introduction

Necrophagous Diptera are of fundamental importance to mankind for four principal reasons: nutrient recycling (Lole, 2005), pollination (Howlett, 2011), transmission of disease (Vinogradova, 2009) and forensic entomology (Brundage, 2011), for which *C. vicina* and *C. vomitoria* are noted as key species in the United Kingdom. *Calliphora vicina* is widely found throughout the UK (Davies, 1999; Hwang & Turner, 2005). Although present in urban areas, *C. vomitoria* is thought to prefer rural areas throughout the U.K. (Erzinclioglu, 1996). Using PCR-RFLP on the COI gene, just 4.3% sequence divergence was identified between *C. vicina* and *C. vomitoria* (Schroeder *et al*., 2003); despite this, the ability of *C. vicina* and *C. vomitoria* to cross has never been researched, though the closely related species *Lucilia sericata* and *L. cuprina* (Diptera: Calliphoridae) are known to produce a hybrid (Waterhouse & Paramonov, 1950). Globally, *C. vicina* is thought to have a wide range, being noted from the sub-Arctic (Nuorteva, 1965) to the sub-Antarctic (Convey et al, 2010). *Calliphora vomitoria* appears to have a range restricted to the Northern hemisphere (Baz *et al*., 2007; Niederegger *et al*., 2010, Iwasa & Inoue 2012). As such, Calliphoriade are an ideal species to observe, due to their wide range of ecological niches occupied, which makes analysis such as latitudinal studies (Saunders & Hayward, 1998), differences in physiology (Slama, 2012) and global genetic studies (Boehme *et al*., 2011) possible.

Diptera are important for nutrient recycling. In the U.K., at one municipal tip alone, it is estimated that 5000 tons of waste arrive per day, of which 2000 tones is putrescible; at peak times an expected 2.16 million *Musca domestica*, *Fannia* sp, and *C. vicina* can be present per month degrading the putrescible waste (Lole, 2005). *Calliphora vicina* are known not only to degrade putrescible waste but they are a known pest within the food industry; in Lofoten, Norway, *C. vicina* are thought to cause some 1-2 million Euros of losses to stockfish industry (Aak, 2011b). Coupled with their role in nutrient recycling is their importance in forensic entomology, as a vertebrate cadaver provides an excellent source of food for over 400 insect species (Payne, 1965). Through an

insect's morphology, age of a maggot can be quite accurately identified (Kaneshrajah & Turner, 2004; Donovan *et al.,* 2006), from which it is possible to surmise a minimum approximate time of death, or post mortem interval (PMI) (Greenberg, 1991). Within the U.K., *C. vicina* and *C. vomitoria* are of fundamental importance due to their rapid colonisation of the corpse (Amendt *et al*., 2004; Erzinclioglu, 1996). The plentiful research on the development rates of many species of Calliphoridae (Davies & Ratcliffe, 1994; Wall *et al*., 1992; Kaneshrejah & Turner, 2004; Donovan *et al*., 2006) and rapid colonization of the cadaver can allow an entomology-based time of death to be more accurate than a post mortem (Greenberg & Kunich, 2002).

Within the European Union, the agricultural value of pollinators was estimated to be €14.2 billion per year in 2008 (Gallai *et al.,* 2009) and with both the growing concern about the loss of pollinators around the world (Kluser & Peduzzi, 2007) and the simultaneous intensification of agriculture (Robinson & Sutherland, 2002), any research surrounding pollinators is very advantageous. Both *C. vicina* and *C. vomitoria* are an often unrecognised resource for pollination (Currah & Ockendon, 1984; Howlett, 2011; Zheng *et al*., 2011). Although there have been no reports of *C. vomitora* being a pollinator of any U.K. crops, given that the physiology of the two species is remarkably similar this is probably due to a paucity of data. A common theme in all Calliphorid pollinator research was that although they were not the most effective of pollinators, due to their high abundance they were the most common visitors, thus pollinated more flowers.

Due to its pre-emptive nature, diapause is of fundamental importance to insects in temperate and colder regions, allowing genetically-programmed developmental changes to occur before the onset of adverse environmental conditions. Whilst in diapause, winter survival is increased through heightened cold tolerance (Saunders & Hayward, 1998; Denlinger, 2002), which can be further increased through acclimation (Pullin & Bale, 1989). Diapause within *C. vicina* is expressed as an elongation of the third larval stage and is dependent on environmental conditions, principally the temperature and photoperiod experienced by the maternal generation (Vinogradova & Zinovjeva,

1972; Saunders, 1987; McWatters & Saunders, 1996). Although there are no direct references to *C. vomitoria* entering diapause in the U.K.; by observing temporal abundance within the species, it is proposed that diapause is entered for approximately 2 months in central London (Hwang & Turner, 2005), although the life stage in which diapause is cued or maintained is not known.

In this study, we sought to describe in more depth the development and physiology of overwintering in *C. vicina* and to determine whether the cues needed for *C. vomitoria* to enter and maintain diapause are similar to those for *C. vicina*. In particular, it is important to describe how environmental conditions influence diapause, as this is crucial to the understanding of the impact of changing conditions (particularly climate change) on their phenology, abundance and distribution (Bale & Hayward, 2010). This research is of importance to the field of forensic entomology, which has largely ignored diapause in analysis. With the advancement of arable farming techniques, crops are being grown throughout the year. It is hoped that this study will assist agriculture research that is seeking to find pollinators with a large temporal range. A further objective was to identify the possibility of a *C. vicina* and *C. vomitoria* cross. The creation of a cross would have significant implications for the field of forensic entomology because the calculation of an accurate PMI is based on a positive identification of the species (Erzincliogl u, 1996).

2.3. Methods

2.3.1. Establishment of cultures

Adult *C. vicina* were collected on the University of Birmingham grounds (52^{o}N; OS SP045838) using a modified baited-target cone-trap, based upon a design by Hwang and Turner (2008).The bottle trap is made from two 2-litre clear, straight-sided plastic bottles. The bottom 8cm of one bottle is removed to form the bait chamber. The collection chamber is formed by inserting the top 8cm of a bottle into the top 30cm of a second bottle (fig. 2.1). The outside of the collection chamber was perforated with many small holes (1mm) to permit air flow which decreases thermal stress on the

insects held within. Entry holes for the flies are created by cutting X shaped slits (1cm in length) in the bait chamber, and folding the four corners inwards. For bait, a plastic weigh boat containing roughly 15g of ox liver was placed inside the bait chamber. A glass vial was attached to the inside of the bait chamber, this contained 15ml of sodium hydroxide and either 5g of ox liver or 5ml of ox blood. The sodium hydroxide and the ox blood acted as an olfactory stimulant and the liver was present as a site for oviposition.

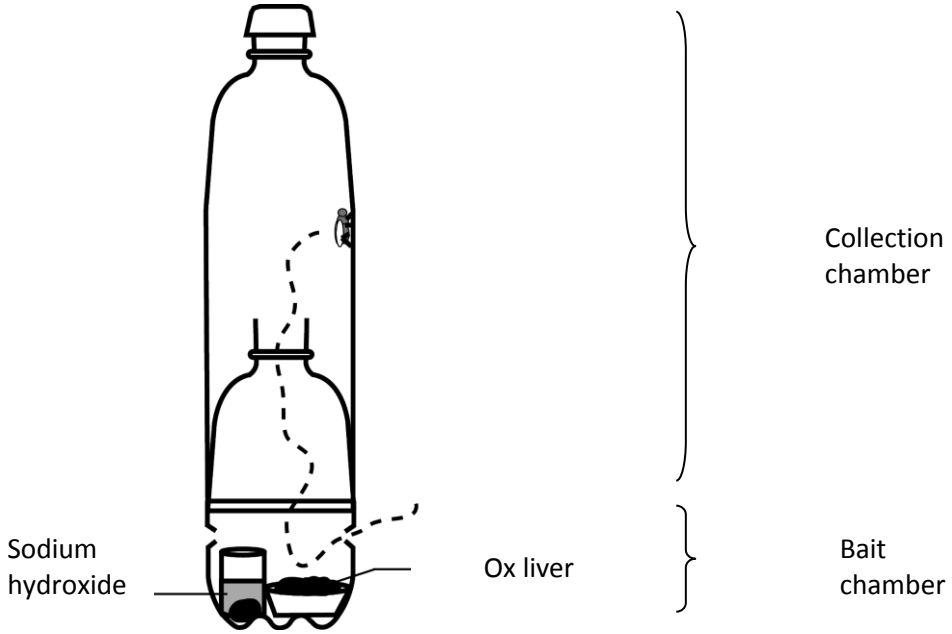


Figure 2.1. Schematic of fly trap used to catch *C. vicina* at the University of Birmingham (52°N). Collection chamber is made of two plastic bottles inserted inside each other. The bait chamber is constructed from the lower portion of a plastic bottle, and holds a vial of sodium hydroxide containing a small amount of ox blood, and a weigh boat containing 15g of ox liver. Figure modified from Hwang and Turner (2005).

2.3.2. Identification.

Traps were placed in a refrigerator (4°C) for 30min to immobilize the insects. Flies were removed from traps and identified using Erzinclioglu (1996); the key differences for the identification of *C.vicina* and *C. vomitoria* are differences in the colours of the jowl, chin bristles and basicosta, as can be seen in figs. 2.2 and 2.3. Once identified, insects were placed in an insect cage; all other insects were frozen.

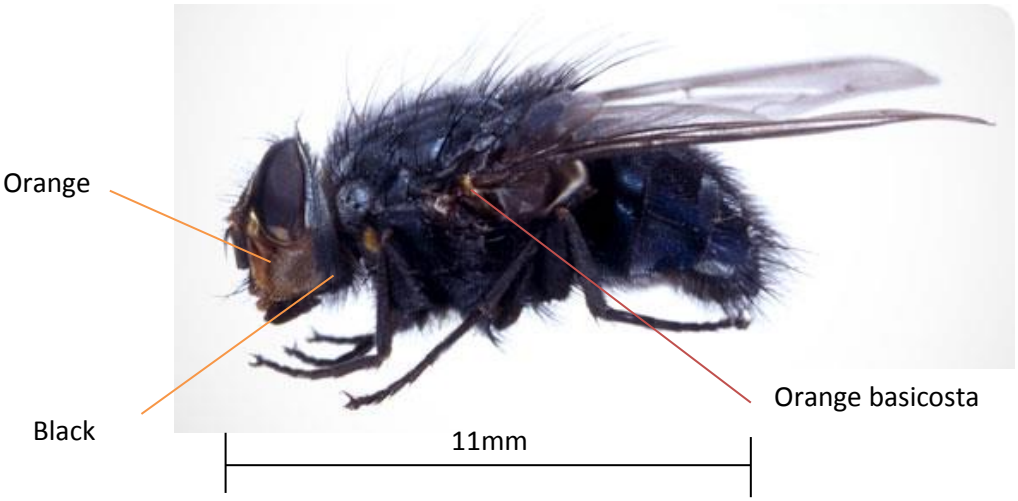


Figure 2.2. Photo of *Calliphora vicina*. Key distinguishing features include the orange jowls, black bristles on the chin and orange basicosta. Photo taken from Natural History Museum, (2011).

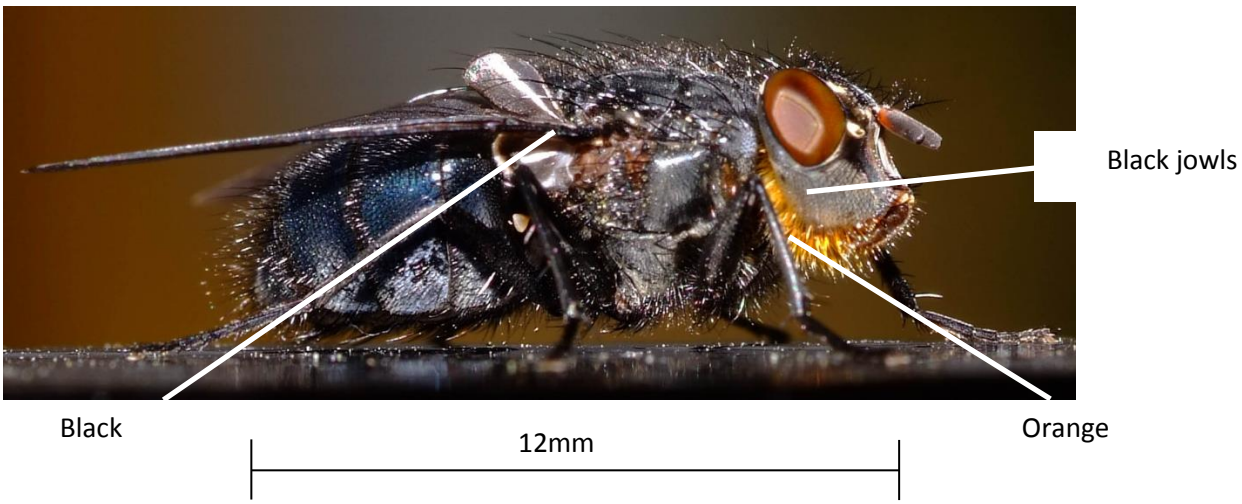


Figure 2.3. Photo of *Calliphora vomitoria*. Key distinguishing features include the black jowls, orange bristles on the chin and black basicosta. Photo taken from Wageninger, 2011

Attempts were made to create a field-fresh population, during weekly trapping during summer months the numbers of *C. vomitoria* were never sufficient to create a population (<1 adults per week, data not included). *Calliphora vomitoria* larvae were obtained from a fishing tackle shop in Birmingham; no prior life history of larvae is known (e.g. latitude of strain, how long cultures have been running or photoperiod experienced). Due to this, only the subsequent generation was used in any experiments.

2.3.3. Culturing

2.3.3.1. Adults

Pupae were placed in a sealed container. On the day of mass eclosion, approximately 300 adults were isolated in cages measuring 30cm on all sides, at 20^{o}C. By using adults from the day of mass eclosion, a synchronous population was produced. Sugar cubes and water were supplied *ad libtum*. 15g of ox liver was supplied for 24h on days 4, 6, 8, post eclosion and every day thereafter up to day (d) 17 (Saunders, 1987). This method was used as it synchronised the oviposition of gravid females so high numbers of eggs were obtained for experimental purposes. Liver was supplied for the first 11 days as a source of protein, for the following days liver was placed as an oviposition site. If oviposition occurred prior to d12 the eggs were discarded, as Saunders (1987) suggested that a minimum of 12d at the required photoperiod is needed to cue the adults for diapause (D).

2.3.3.2 Programming of diapause and non-diapause larvae

In *C. vicina,* adults held at a photoperiod below their CDL (16.5h of light at 51^{o}N- McWatters & Saunders 1996) will enter diapause (D) and those held above their CDL will be non-diapause (ND). Accordingly, cages were either placed inside incubators or in controlled temperature rooms at one of two photoperiods. Adults experiencing a 'long day' (LD) light:dark 18:6 produce ND L3 progeny, while adults maintained under 'short day' (SD) conditions light:dark 12:12, produce D L3 progeny, under appropriate temperature conditions.

2.3.3.3. Larvae

Once a suitable egg mass had been oviposited on the liver during a 24 h period (>300 eggs), eggs were removed and placed in constant darkness (DD), in an airtight plastic box lined with wet tissue paper (100% relative humidity). A high humidity environment was chosen as low humidity levels cause the chorion (egg shell) to change shape, which results in lower hatching success (Davies, 1950). After 24h at 20^{o}C, 300 hatched L1 larvae were transferred onto an excess of liver at 11^{o}C

DD. Mature larvae were provided with ~5cm of dry sawdust in which to continue development (enter diapause or from puparia), once active feeding ceases. To ensure that only larvae that had exited on the day of mass wandering would be used, the sawdust was sieved daily and larvae which exited the foil parcel (wandered) before or after the day of mass wandering were removed.

2.3.4. The effect of species and photoperiod on time to larval wandering

To determine whether photoperiod had an effect on the number of days to wandering, adults of *C. vicina* were allowed to oviposit upon liver and the number of days taken for larvae to cease active feeding and wander off the larvae was recorded. Adults used in experiments had experienced either an LD or SD photoperiod. Sawdust was sieved daily for wandering larvae in all cases and the number days from oviposition to mass wandering recorded.

2.3.5. Diapause incidence and duration in C. vicina and C. vomitoria

2.3.5.1 Effect of photoperiod on timing of pupariation

Diapause incidence and duration in both species can be determined by observing larvae from oviposition to pupariation. For *C. vicina*, Saunders (1987) indicated that if larvae pupated in less than 30 days, they had not entered D, as such, D is indicated by a lack of pupariation by d30 post oviposition. For *C. vomitoria*, there is no literature on the successful rearing of D larvae, the only reference to larvae diapause was proposed by Nesin (2011 – personal communication), who suggested that eggs should be placed at 11^{o}C for 8d, after which larvae should be transferred to 5^{o}C until their gut contents have been evacuated. After this they should be stored at 1^{o}C; which should result in D larvae that are capable of surviving up to 6 months in an airtight container. This, however, does not prove diapause, as the threshold temperature for development is 6^{o}C (Higley & Haskell, 2001), well above the suggested culturing temperature of 1^{o}C. Thus the extended time spent to develop is almost certainly due to larvae being held some 5^{o}C lower than their minimum

temperature for development, rather than diapause being entered. As such, SD and LD adults of both species were reared as protocol in section 2.3.3.1 (*C. vicina* SD n=13222; *C. vicina* LD n=913; *C. vomitoria* SD n=1685; *C. vomitoria* LD n=2440). Subsequent larvae were stored at 11°C and the percentage that remained as larvae at d30 recorded.

2.3.5.2. Effect of larval temperature on timing of pupariation

Vaz Nunes & Saunders (1987) highlighted that *C. vicina* larvae exposed to temperatures over 15°C, regardless of the photoperiodic regime of their parental generation, would universally terminate D and pupariate. The alteration of D incidence at low temperatures has never been looked at in either species. Two larval rearing temperatures were compared for larvae of *C. vicina* and *C. vomitoria*. Larvae were all progeny of SD adults held at 20°C. In *C. vomitoria,* after eggs hatched, larvae were held at 11°C for 8 days (see section 2.3.3.2. for further details); afterwards larvae either remained at 11°C (n=80) or were transferred to 5°C (n=30). *Calliphora vicina* were held at 11°C for 30 d; after which larvae either remained at 11°C (n=80) or were transferred to 5°C (n=30). The percentages of insects remaining as larvae were recorded on d30, 40, 50 and 60.

2.3.6 Effect of moisture on timing of pupariation within C. vomitoria

During initial culture of *C. vomitoria* it was noted that larvae that had been placed in dry sawdust were pupariating earlier than their *C. vicina* counterparts. The ability of *C. vomitoria* to diapause in low humidity environments was further questioned when Nesin (2011, personal communication) suggested that larvae should be stored in moist environments at low temperatures to allow diapause to progress.

Adults and larvae of *C. vomitoria* were cultured as described in method section 2.3.3. On the day of mass wandering, three replicates of 20 larval progeny from SD adults were placed inside ventilated containers holding a ratio of water to sawdust of either 0:1, 1:1 or 2:1. Larvae were placed in an

incubator at 5°C, due to larvae pupariating if held at higher temperatures, and the percentage of larvae pupariated was recorded daily; the percentage surviving to eclosion was also recorded.

2.3.7. Hybrid of C. vicina and C. vomitoria

Waterhouse & Paramonov (1950) suggested that the two closely related Calliphoridae, *Luciia cuprina* and *L. sericata,* were capable of creating a viable cross. The successful hybridization of *C. vicina* and *C. vomitoria* has never been reported. To determine whether the successful hybridization of *C. vicina* and *C. vomitoria* could occur, pupae of *C. vicina* and *C. vomitoria* were individually housed in separate 1.5ml Eppendorf® at 20°C, until eclosion. After eclosion, the species was confirmed using keys by Erzinclioglu (1996) and each adult fly was sexed. Two cages of adults were then created: the first holding female *C. vicina* and male *C. vomitoria* (termed P ♀ *C. vicina*), the second holding male *C. vicina* and female *C. vomitoria* (termed P ♀ *C. vomitoria*). Each cage contained roughly 70 female and 70 male flies. Cultures were reared according to standard protocol and the percentage of eggs which successfully eclosed was recorded. To determine whether if the F1 generation could successfully interbreed and result in viable offspring, the F1 larvae of P ♀ *C. vomitoria* were allowed to interbreed to create F2 larvae.

2.3.7.1. Phenotype of parental, F1 and F2 hybrids

In order to determine which morphological phenotypic characteristics the P ♀ *C. vomitoria* had inherited, the incidence of 3 key criteria (basicosta, cheek and chin bristle colour), usually used to distinguish between these species were noted; these features can be seen in figs. 2.2 and 2.3. The gender of parental, F1 and F2 generations was also assessed.

2.3.8. Statistical tests

All data were subjected to a Levenes test for equal varience and a Kolmogorov-Smirnov test for equal distribution to ensure the correct statistical test was employed. The differences between the number of days taken for larvae of *C. vicina* and *C. vomitoria* (LD and SD photoperiods) to wander

were examined using a Student's T-test (Excel, 2010, version 14.0.6123.5001) as was the effect of larval temperature on timing of pupariation. To examine whether the effects of photoperiod on the timing of pupariation are statistically different an ANOVA was performed (Predictive Analytics SoftWare, PASW statistics 18) once data were found to have normal variance and distribution. The relationship between the proportions of female adults in populations of *C. vicina, C. vomitoria* and their hybrid (F1 and F2 generations) was examined through the use of a G-test (Microsoft Excel, 2010).

2.4. Results

2.4.1. Differences in culturing

The following differences were noted between *C. vomitoria* and *C. vicina*:

1. *Calliphora vomitoria* larvae had the tendency to spend an greater proportion of their time during L1 and L2 stages off the liver, wandering around the foil parcel or box; this time was increased if larval numbers were high (>200), or if the foil parcel was closed.

2. The mean number of days to wandering for LD *C. vicina* was 8.4 (fig. 2.4). This was significantly shorter than the 11.5d to wandering for SD larvae ($P<0.001$). Both LD and SD *C. vomitoria* larvae had significantly longer wandering periods than *C. vicina* at 18.5 d and 16.6 d respectively (fig. 2.4) ($P<0.001$ for both photoperiods). The difference in wandering time between LD and SD *C. vomitoria* larvae was not significant ($P=0.26$). Mass wandering occurred over a period of time (3-4d) in *C. vomitoria* and just one day in *C. vicina*.

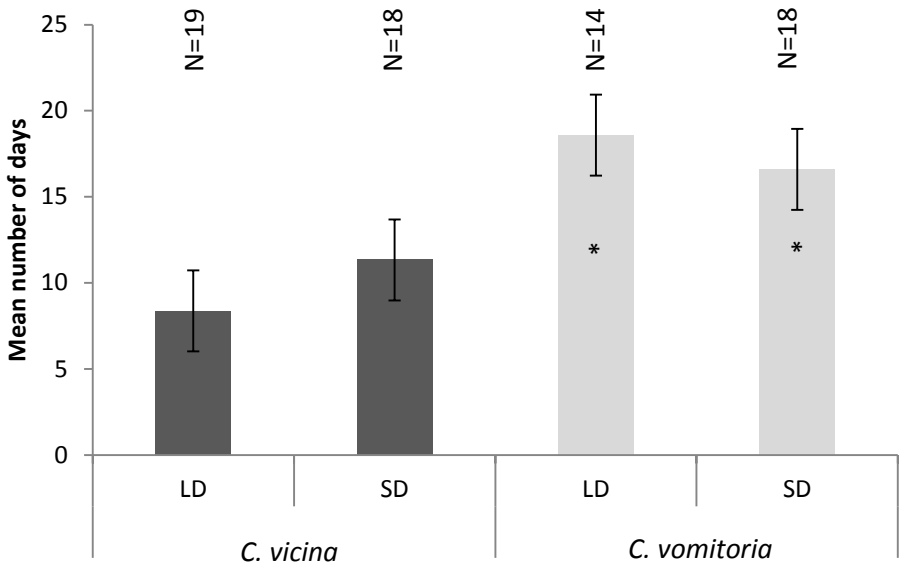


Figure 2.4. Mean number of days (±SE) for larvae to cease active feeding (wander) after oviposition, when held at 11°C. Adults of *C. vicina* and *C. vomitoria* were placed in a cage held at 20°C and exposed to either a long day (LD) or a short day (SD) photoperiod. Larvae were monitored daily and the day of mass wandering (exit off liver) was noted. A total of 19 mass wandering events were recorded for LD *C. vicina*, N=18 SD *C. vicina*, N=14 LD *C. vomitoria* and N=18 SD *C. vomitoria*. Groups that are not significantly different are marked with asterisk.

2.4.2. Diapause incidence and duration in C. vicina and C. vomitoria

2.4.2.1. Effect of photoperiod on timing of pupariation

Photoperiod had a clear effect on timing of pupariation in *C. vicina* (fig. 6), with 77.2% of SD *C. vicina* remaining as larvae 30d post oviposition, and just 14.4% of *C. vicina* remaing as larvae under LD conditions. This difference was statistically significant ($t=10.889$, d.f.=19, $P<0.001$). *Calliphora vomitoria* showed a very different response, with adult photoperiod appearing to have no significant effect on the proportion remaining as larvae; at 30d post oviposition it was 9.1% for SD and 2.7% LD cultures ($t=1.646$, d.f.=17, $P=0.118$).

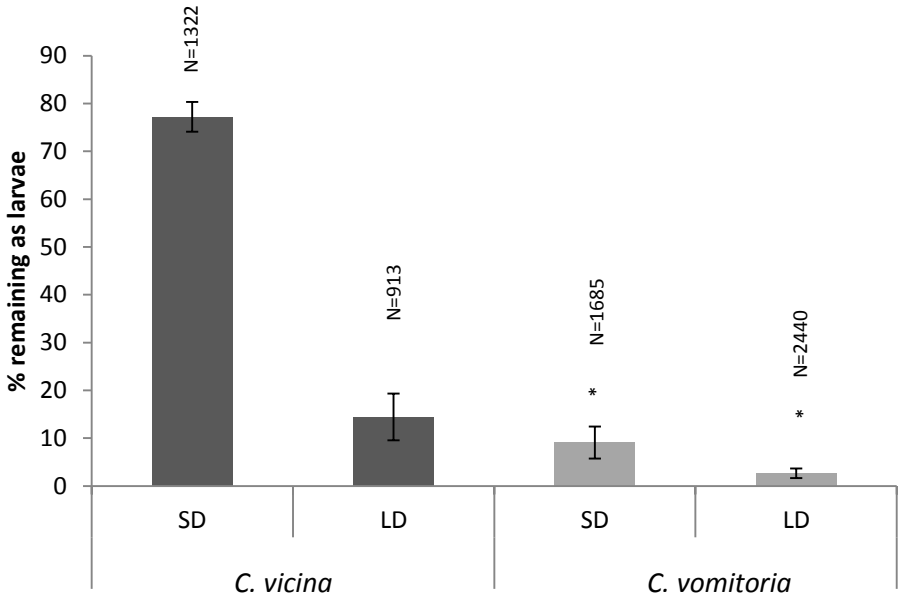


Figure 2.5. Mean percentage (±SE) of individuals remaining as larvae 30d post oviopsition in *C. vicina* and *C. vomitoria*. Two photoperiods were established for adult cultures, short day (SD) and long day (LD). Larval progeny were held at 11°C until day 30 post oviposition, when the proportion remaining as larvae was calculated. Groups that are not significantly different are marked with asterisk.

2.4.2.2. Effect of larval temperature on timing of pupariation in SD cultures

In both *C. vicina* and *C. vomitoria,* the percentage of individuals remaining as larvae decreased with time (fig. 2.6). This was most marked for *C. vomitoria,* which saw a rapid decrease between 30 and 50d post oviposition, particularly when larvae were held at 5°C (from 100% at d30 to 10.4% at d50). Larvae of *C. vomitoria* held continuously at 11°C rapidly pupariated, with 10.3% remaining as larvae at d30 post oviposition and just 0.6% remaining at d40. *Calliphora vicina* generally saw a higher proportion of individuals remaining as larvae than *C. vomitoria*, the only exceptions being for cultures held at 5°C when assessed at 30d and 40d post oviposition. This decrease in the proportion of individuals remaining as larvae between d30 ($P<0.001$) and d40 ($P=0.03$) was significant both for larvae held at 11°C and larvae held at 5°C.

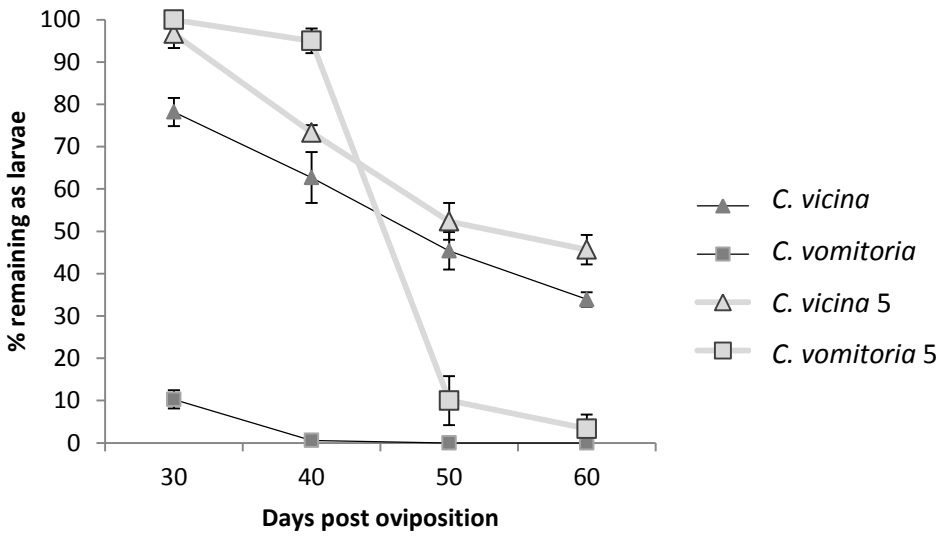


Figure 2.6. Mean percentage (±SE) remaining as larvae at 30, 40, 50 and 60d post oviposition for *C. vicina* (diamond) and *C. vomitoria* (square). Larvae were held at 11°C (darker lines, n = 80) or at 5°C (lighter lines, n=30) in constant darkness. Larvae used were progeny of adults held at an SD photoperiod.

2.4.3. Effect of moisture on timing of pupariation and survivorship within C. vomitoria

High moisture levels in the larval substrate (sawdust) delayed the timing of pupariation in *C. vomitoria* (fig. 2.7). The timing to 50% pupariation was different in all groups; the low moisture group (0:1 water: sawdust) resulted in the lowest time at 43.1d (95% fiducial limits 42.1 and 43.9), larvae held in an equal weight of water to sawdust saw 50% pupariation in 49.5d (95% fiducial limits, 48.7 and 51.4) and larvae exposed to a weight ratio of2:1 water to sawdust pupariated in 51.2d (95% fiducial limits, 50.0 and 51.9). Timing of pupariation could thus be seen to be delayed in media where the mass of water exceeded that of sawdust (as represented by non-overlapping fiducial limits). Beyond this level, up to 2:1, there was no significant additional delay. Low moisture (0:0 water : sawdust) lead to high mortality (53.3 ± 6.0%), larvae held in 1:1 suffered 3.4 ± 1.2% mortality and no mortality was recorded when larvae held in 2:1 (water: sawdust).

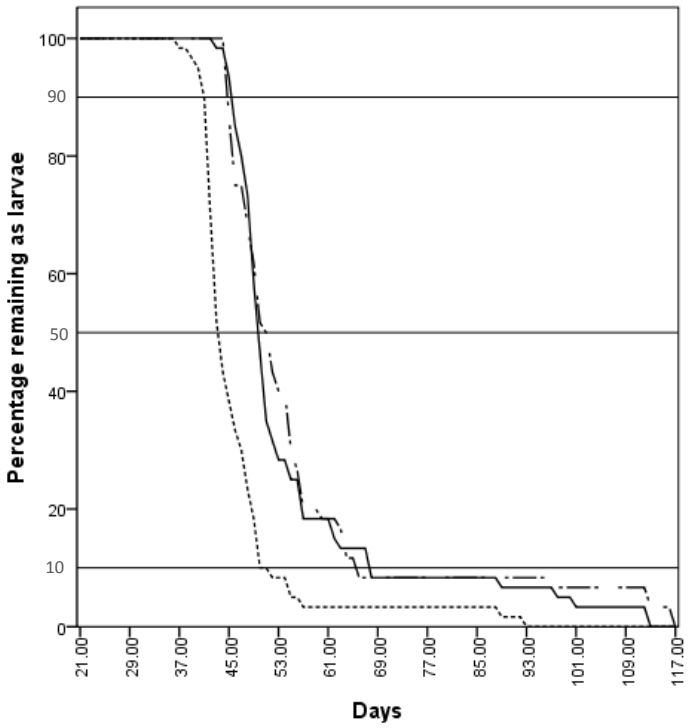


Figure 2.7. The effect of moisture level on the number of days to oviopsition in *C. vomitoria*. Larvae were progeny of short day adults (held at 12:12 photoperiod) and cultured at 20°C; subsequent larvae were held at 11°C until 2-3 days before wandering and then held at 5°C. On the day of mass wandering, three containers each containing 20 larvae were either placed in media with a water:sawdust mass ration of 0:1, 1:1 or 2:1 at 5°C. The numbers of pupae and larvae were recorded daily.

2.4.4. Crossbreeding of C. vicina and C. vomitoria

Crossing ♀ *C. vicina* with ♂ *C. vomitoria* did produce eggs, though these did not hatch in to L1 larvae. Crossing ♀ *C. vomitoria* with ♂ *C. vomitoria* however, produced some viable offspring (16.7 ±2.1%, n=467). This cross also produced a viable F2 generation (98 ± 1.4%, n=30). Morphological assessment of the F1 and F2 generations showed that adults had *C. vomitoria*-like features (basicosta, chin hair, and cheek colour; see figs. 2.2 and 2.3). Overall the gender ratio did not significantly differ between hybrid or parental generations (P=0.315), with the F1 and F2 crosses having a similar gender ratio (32.5 and 40% female respectively) to *C. vicina* (42.5%, fig. 2.9).

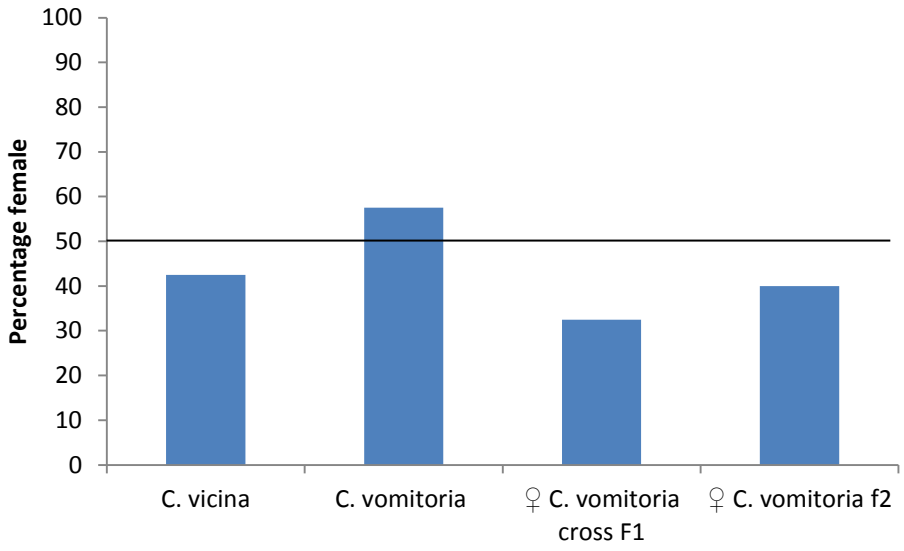


Figure 2.9. Mean percentage of population that were female for *C. vicina*, *C. vomitoria*, F1 and F2 P ♀ *C. vomitoria* (n=40 for all samples). As the frequency of females was recorded from lone populations, standard error bars could not be produced.

2.5. Discussion

Although field-fresh *C. vicina* cultures were readily established, attempts to create a field-fresh *C. vomitoria* culture were unsuccessful, during monthly replenishment of cultures no more than two *C. vomitoria* were ever trapped in one month. In the wild, *C. vicina* is noted as being synathropic (closely associated with humans); as such they are a common insect throughout most urban areas in the United Kingdom (Erzinclioglu, 1996). *Calliphora vomitoria* is more common in rural areas (Gennard, 2008), prefering dense woodland cover for optimal breeding conditions (MacLeod & Donnelly, 1957; Nuorteva & Laurikainen, 1964). To the best of knowledge only two papers have reported catching *C. vomitoria* in small baited traps, Brundage *et al.* (2011) used a commercial fly trap baited with 6oz of beef liver, Hwang and Turner (2005) used a self-made baited trap with 15g of ox liver, it was this design that was used in the present research. Davies (1999) placed 61 mice in various locations around Durham and found although *C. vicina* were present no *C. vomitoria* oviposited upon the mice, though *C. vomitoria* did colonise sheep carcasses, more so in the field site than the garden site. The remainder of the papers used whole pig carcasses in woodland settings to obtain samples (Reibe *et al*., 2009;

Matuszewski *et al,* 2010; Neideregger *et al*., 2010; Sabanoglu & Sert, 2010). The combination of the urban nature of the collection site and the small size of the oviposition medium in the traps is thought to be the reason for the low number of *C. vomitoria* caught. Due to the high density of people on the university grounds and the close proximity of the trapping site to lecture theatres/residential areas, requests for large carrion (pig) baited traps were hastily denied. Efforts to find a more rural trapping site at the same latitude as the University of Birmingham were hampered by lack of response by land owners. This is why *C. vomitoria* cultures were established from shop bought maggots. The use of shop bought maggots is not without problems, as the origin is unkown, as is the thermal and photoperiodic regimes used to rear the flies. When hatched the maggots were of mixed species (*Lucillia* spp. and *Calliphora* spp.) and mainly contained *C. vomitoria* and *C. vicina*. Here we consider whether these differences in habitat and biology might explain some of the differences identified when culturing these species.

Data presented here on the effect of photoperiod on diapause incidence in *C. vicina* agrees with the findings of McWatters and Saunders (1996), who identified that LD conditions (18:6 photoperiod) resulted in a high percentage of larvae pupariating, and therefore a low incidence of D (c. 10%), whereas SD conditions resulted in a high incidence of diapause (77.2%). In this study, diapause incidence in LD conditions was 14.4% (fig. 2.5), whereas 77.2% of larvae entered D under SD conditions (fig. 2.5). This was not seen in *C. vomitoria* where both SD and LD photoperiods resulted in over 90% of larvae pupariating before 30 days post oviopsition, when held at 11^{o}C. When larvae were held at the lower temperature of 5^{o}C, both species saw a higher proportion remaining as larvae at day 30 post oviopsition. Due to the poikilothermic nature of insects the developmental rate is closely linked to temperature, therefore the increase in the proportion remaining as larvae is thought to be due to a delay in development rather than to an increase in diapause incidence. *Calliphora vomitoria* subsequently saw a sharp decrease in the number remaining as larvae after 40d post oviposition, further suggesting that there is a delay

of development by at least 10d, rather than the data indicating that larvae are entering diapause. Despite the brief mention of diapause being induced *C. vomitoria* by Vinogradova (1986), and Nesin (2011, personal communication), the cues required to induce diapause could not be revealed in this strain of *C. vomitoria*, with adults not being able to cue diapause for the two photoperiodic regimes administered in this study (12:12 and 18:6, light : dark). This may be due to the photoperiod not being short enough; alternatively the adult stage may not be the sensitive stage in *C. vomitoria*. A third alternative is the possibility that the *C. vomitoria* strain, assumed to be from either Leningrad (60^{o}N) or Gorky (53^{o}N), used in studies by Vinogradova (1986) diapause under different conditions than strains originating from the UK. This trait has been found in *C. vicina,* in which a northern strain from 65^{o}N has a CDL 2h longer than that of the southern strain from 51^{o}N; these strains were also found to respond differently to temperature, with the northern strain being unresponsive to temperature when exposed to short days (McWatters & Saunders, 1998). The lineage of *C. vomitoria* used in this study was not known, as such neither are the photoperiodic or thermal conditions that the previous generations were raised in. This is of concern as unplanned selection for non-diapause larvae may have taken place; the selection for non-diapause allowed Denlinger (1979) to alter the proportion of *Pseudosarcophaga spilogaster* entering diapause from 80 to 10% in just 10 generations. With neither the number of generations nor the culturing methods known, the possibility of unplanned selection can neither be confirmed nor ruled out.

The culturing of *C. vicina* and *C. vomitoria* followed similar protocols, although *C. vomitoria* tended to spend a reduced proportion of its first three life stages actively feeding, which may explain the increased time to wandering. Alternatively, it may be due to *C. vomitoria* weighing more than *C. vicina,* with ND L3 *C. vomitoria* on average weighing 107mg (12.5%) more than *C. vicina* (data not presented). Thus, the increase in time to wandering may be due to an overall increase in amount of time feeding. The increased time to wandering in *C. vomitoria* was also noted by Block *et al.* (1990).

Moisture had a large effect on *C. vomitoria,* with larvae reared in dry sawdust exhibiting a high mortality rate (53.4%) and a significantly decreased time to 50% pupariation, 6 days less than *C. vicina,* when held at 5^{o}C. Variations in precipitation have a large effect on insects (Benoit, 2010) and are thought to, at least partly, define the species range (Yoder *et al.*, 2011). Although this is the first published research examining the importance of environmental water on pupariation and survivorship in *C. vomitoria,* the methods used in prior published research suggest that larvae were successfully reared in dry sawdust when held >20^{o}C (Block *et al*., 1990; Bowen *et al.,* 1996; Wooldridge *et al.,* 2007). The low eclosion success of *C. vomitoria* when held at low humidity levels is of concern in the light of global warming, as mean winter temperatures are predicted to rise and episodes of drought are predicted to become more frequent (Breshears *et al*., 2005). An increase in temperature may have significant effects due to a consequent increase in metabolic rate (Dillon *et al*., 2010), which will increase respiratory water loss through an increase in gas exchange (Woods & Smith, 2010); this, combined with increased drought episodes, could result in very high field mortality. Survival has been seen to be influenced by soil moisture in the Dipteran blueberry maggot, *Rhagoletis mendax*, which saw a decrease in survival rates as soil moisture was increased, in both field and laboratory samples (Renkema *et al*., 2012). An opposing trend was noted in the Queensland fruit fly, *Bactrocera tryoni,* which saw 85% mortality when held at 0% soil moisture and less than 10% mortality when held in soil moistures between 10 and 75% (Hulthen & Clarke, 2006); most interestingly, they suggested that prepupal wandering larvae were able to sense soil moisture, indicated by an increased percentage of larvae pupating in soils at 75% humidity rather those at either humidity extreme (0 and 100%). Thus, if drought occurs due to climate change, larvae of *C. vomitoria* could move to an area of higher soil moisture, assuming they also possess the ability to sense soil moisture, as was seen in *B. tryoni*.

The evolution and colonization pattern of *C. vicina* and *C. vomitoria* have not been extensively researched. Initial studies by Schroeder *et al.* (2003) used PCR-RFLP (restriction fragment length

polymorphism), which showed just 4.3% sequence divergence between the two species (15 nucleotide substitutions) in a 349 bp region of the COI gene. As the COI region is known to be a highly variable region, this suggests that *C. vomitoria* and *C. vicina* are closely related, which may explain why crossing these two species created a viable hybrid. This is not the first hybrid to be seen within Calliphoridae. The positive laboratory hybridization of *L. cuprina* and *L. sericata* was first described in 1950 (Waterhouse & Paramonov, 1950); results suggested that only a one way cross was possible (male *L. cuprina* and female *L. sericata*) and, as in this study, there were difficulties in creating a viable hybrid. Waterhouse and Paramonov (1950) suggested that hybridization in the field was unlikely to occur at a significant frequency due to low viability, as seen in the laboratory. The first possible evidence of hybridization in the field was noted in 1996 (Stevens & Wall, 2006) after random amplified polymorphic DNA (RAPD) and complimentary mitochondrial DNA (mtDNA) was analysed from insects from 17 different sites worldwide. *Lucilia cuprina* from Oahu, Hawaii appeared to have mtDNA similar to *L. sericata* whereas RAPD grouped Hawaiian flies with *L. cuprina* (Stevens & Wall, 1996); this was later confirmed using complimentary nuclear (28s rRNA) and mitochondrial (COI and COII) gene markers (Stevens *et al*., 2002).

When male *C. vomitoria* and female *C. vicina* were crossed, the resultant eggs were found to be not viable, unlike the opposite cross. This may be due to the bacteria *Wolbachia,* which is known to be present in over 65% of insect species (Hilgenboecker *et al*, 2008) including the closely-related blowfly *Protocalliphora* (Whitworth *et al.,* 2007). *Wolbachia* is an intracellular organism which is able to exert an effect on the reproductive output of its host. Although *Wolbachia* is generally associated with feminization, parthenogenesis or male killing, all of which result in a near-complete female population being produced, *Wolbachia* most commonly results in cytoplasmic incompatibility (CI) (O'Neill *et al*., 1992). CI occurs when insects do not harbour the same strain of *Wolbachia*; sperm from infected males are incompatible with eggs from females which are infected with a different strain of the bacterium (see review – Werren *et al*., 2008). It

creates this incompatibility at in a two stage process: first, the correct formation of sperm during spermatogenesis is disrupted, but then, eggs which harbor the same strain are able to rescue the modified sperm and thus allow fertilization to occur (Werren, 1997). Application of two antibiotics, tetracycline and rifampicin, can eliminate *Wolbachia*. However, for some species, such as the Collembolan *Folsomia candida* (Pike & Kingcome, 2009) and the Hymenopteran *Asobara tabida,* elimination of bacteria leads to sterility because *Wolbacia* acts as an obligatory parasite preventing cell death and enabling successful oogenisis (Dedeine *et al,* 2001).

The COI gene is known to be a highly variable region and is often used to examine intraspecific variation within species, e.g. geographic variation between strains, including in Calliphoridae (Harvey *et al*., 2008). The presence of *Wolbachia* is known to decrease the effectiveness of using COI as a method of DNA barcoding (Whitworth *et al.,* 2007); in their study COI barcoding was found to give a very high error rate when barcoding *Protocalliphora* species, 80% of which were shown to harbor the *Wolbachia* parasite. Thus, if *Wolbachia* are present in *Calliphora sp.,* the use of COI as a barcoding system for the correct identification of larvae, as was suggested by Harvey *et al.* (2008), may be futile.

The correct identification of species is of great importance in the field of forensic entomology. This is due to different species having different development rates, which must be known for the calculation of an accurate PMI (Amendt, *et al*., 2004). For example, when reared at 20^{o}C, it takes *C. vicina* 538.4 h to complete development from egg to pupae, whereas *C. vomitoria* are known to take 572.6 h at the same temperature (Ames & Turner, 2003). This would lead to an inaccuracy of 34.2 h if the species was misidentified. The ability of *C. vicina* and *C. vomitoria* to produce a viable hybrid raises questions regarding the accuracy of samples collected from the field exhibiting *C. vomitoria*-like morphology (chin hair, cheek and basicosta colour) as the development rate of the hybrid has never been researched.

In this research, it was noted that when female *C. vomitoria* and male *C.vicina* were crossed, resultant hybrid adults had morphological features similar to *C. vomitoria*, i.e. morphology was inherited maternally. This was not noted in the hybrid of male *L. cuprina* and female *L. sericata* in which hybrids showed mixed morphological features (Waterhouse & Paramenol, 1950). The hereditary of diapause in *C. vicina* is known to be entirely maternally regulated and diapause duration was seen to be influenced by both male and female parents (McWatters & Saunders, 1996). A limited number of cold tolerance experiments have been conducted on *C. vicina* and *C. vomitoria* (Block *et al*., 1990; Saunders and Hayward, 1998), though a direct comparison of cold tolerance in these species would be very interesting, furthermore, the analysis of cold tolerance in hybrid lines may further the knowledge of the hereditary of cold tolerance phenotypes.

3. Effect of water on the cold tolerance strategies of two Calliphoridae species

3.1. Summary

Cold hardiness enables many insects to cope with both seasonal and more rapid fluctuations in temperature, which allows populations to increase survival and develop to the next generation, both within and between seasons. Insects can be classified in to a number of different cold hardiness categories dependent on their ability to survive at cold temperatures. In this study, the cold hardiness strategy of *C. vicina* (R-D.) and *C. vomitoria* (L.) was compared in light of the habitat in which resides.

Of all life stages, eggs had the lowest SCP in both species, at c.-25^{o}C. Cold resistance was modest in other stages (range -10.4^{o}C to -20.8^{o}C in *C. vicina* and -7.7^{o}C to -16.0^{o}C in *C. vomitoria*). In all but L3 larvae (early – whilst actively feeding) the SCP of ND *C. vicina* was significantly lower than that of *C. vomitoria*.

Freeze tolerance of both *C. vomitoria* and *C. vicina* was defined as survival of their SCP after being inoculated with ice. Although *C. vicina* showed a limited ability to survive until pupariation (18.8%), none survived to eclosion. A considerable number (49.1%) of *C. vomitoria* were able to survive to eclosion and 16.2% were even able to survive 1hr frozen at -5.5^{o}C.

A rapid cold hardening response was elicited in *C. vomitoria* with ND larvae showing 19.2% survival after a 2hr plunge at -13^{o}C (DTemp). Acclimation treatments were established by either holding at 0^{o}C, -2^{o}C or -5^{o}C for 2 hours followed by a plunge to DTemp, or alternatively a ramped acclimation from 11^{o}C to -5.5^{o}C at 0.5^{o}C min^{-1} before being held at DTemp. Survival rate was highest in larvae acclimated for 2h at -5^{o}C, in which survival increased to 77.5%. A comparison to *C. vicina* is made below.

3.2. Introduction

Temperature is known not only to affect the induction and initiation (McWatters & Saunders, 1998) of diapause (Kimura & Minami, 1980), by can affect insects in numerous ways, influencing development, reproduction and longevity (Tauber *et al.* 1986). During extremes of temperature, survival is dependent upon the temperature and duration of exposure and its interaction with the cold hardiness of the individual (Bale, 1987). Bale (1987) defined cold hardiness as the characteristics of an individual that prevent harmful effects when held at low temperatures. The applicability of the cold tolerance strategy employed by an insect to its environment is known to affect overwintering survival rates (Bale, 1987; Chen *et al.*, 1991a; Pullin, 1996).

Two primary different cold hardiness strategies have been identified in insects: freeze intolerance (or freeze avoiding – cannot survive internal ice formation) and freeze tolerance (can survive internal ice formation), with freeze intolerant insects being sub-categorised as either chill intolerant or chill tolerant. For chill-intolerant insects, mortality can be sustained as a result of experiencing a chilling injury at temperatures above 0^{o}C. For example, the greater wax moth, *Galleria mellonella* experienced more than 20% mortality when 2 day (d) old eggs were transferred from 30^{o}C to 5^{o}C, and when transferred to 0^{o}C, mortality increased to 100% (Roversi *et al.,* 2008). Freeze intolerant species are able to seasonally depress the temperature at which their internal fluids will freeze; the point at which freezing occurs is termed the supercooling point (SCP). The pistachio fruit hull borer, *Arimania comaroffi*, is one such example of a freeze intolerant insect which can seasonally depress its SCP (Bemani *et al*., 2012). Freeze tolerant species, for example the Arctic midge, *Belgica antarctica* (Hayward *et al.,* 2007), can tolerate internal ice formation in extracellular spaces. Extracellular freezing is often promoted at higher sub-zero temperatures by ice nucleating agents although survival at temperatures well below the SCP has been recorded without nucleator activity in the haemolymph, in the freeze tolerant

hoverfly, *Syrphus ribesii* (Hart & Bale, 1997). Insects can also increase their survival through accumulation of cryoprotectants (Duman *et al.,* 1991; Lee, 1991).

As insects are poikilotherms, internal body temperature closely tracks external temperature. When the temperature drops below zero, ice should form, but insects can differentially accumulate solutes in their haemolymph (e.g. ions, amino acids, sugars and proteins) that depress the point at which freezing occurs (Hou *et al.,* 2009; Bemani *et al.,* 2012). This temperature can be further depressed through the reduction of water volume, as smaller volumes of water have a lower freezing temperature (Larsen & Lee, 1994). Freeze intolerant species often have a lower SCP than freeze tolerant insects, as the SCP for freeze intolerant species represents the lowest temperature at which survival is possible. For freeze tolerant species, the formation of internal ice represents a dramatic shift in physiology, with radical decreases in metabolic rate, water loss (Irwin & Lee, 2002) and removal of metabolic by-products, together with increased anoxia (Storey & Storey, 1988). Due to the suppression of metabolic rate and water loss, insects are able to increase survival by entering a frozen state, thus the SCP is often high in freeze tolerant insects to enable insects to freeze at a higher temperature. To further increase the temperature at which freezing occurs, ice nucleating agents (INA's) can be employed. There are many types of ice nucleating agents, and each have different efficacy; for example, ice nucleating proteins often allow the formation of ice crystals from -6^{o}C to -9^{o}C (Zachariassen & Hammel, 1976), ice nucleating fungi form ice at around -5^{o}C (Tsumuke *et al*., 1992) and inoculation by external ice typically forms ice crystals at -1^{o}C (Tursman *et al*., 1994).

Insects in the temperate zone typically enter diapause to allow survival over winter (Tauber *et al*., 1986; Fields *et al*., 1998). Temperate insects which enter diapause are able to enhance their cold tolerance over winter (Li *et al*., 2000, Goto *et al.,* 2001); for example, the pine caterpillar, *Dendrolimus tabulaeformis*, is able to increase survival at -10^{o}C, -17^{o}C and -21^{o}C if larvae are in

diapause (Han *et al.,* 2005). Insects are also able to improve their cold tolerance as they progress from summer through to winter; for example, the rice stem borer, *Chilo suppressalis*, is able to increase its survival of 24h at -15^{o}C from <5% in insects collected in October, to 100% in insects collected in January (Ishiguro *et al*., 2007), furthermore, cold tolerance was seen to increase after acclimation. A similar acclimation response was observed in the common hoverfly, *Syrphus ribesii,* which shows 0% survival to -15^{o}C for 1 min when not acclimated, but 100% survival after repeated periods of acclimation at 0^{o}C (Hart & Bale, 1998). This seasonal improvement in cold tolerance in preparation for winter is activated mainly by temperature and photoperiod (Tauber *et al.,* 1986*;* Lee, 1991) over the preceding weeks or months. Insects are also able to rapidly increase their cold tolerance after a short exposure (minutes or hours) to a less severe pretreatment, through rapid cold hardening (RCH); this can be seen in both diapause and non-diapause states. For instance, non-diapause eggs of the yellow spotted longicorn beetle, *Psacothea hilaris*, are able to increase their ability to survive a 2h treatment at -22^{o}C by 30% if they first receive a pretreatment at 0^{o}C for 4h (Shintani & Ishikawa, 2007).

This study aims to conduct a detailed investigation into the cold tolerance strategies of two Calliphoridae species, *C. vicina* and *C. vomitoria* and also their cross (chapter 2). The cold tolerance strategy of *C. vicina* has been established (Saunders and Hayward, 1998), however, the cold tolerance strategy of *C. vomitoria* is less well studied. The SCPs of both *C. vicina* and *C. vomitoria* at all life stages were examined. This furthered research by Block *et al,* (1990) who examined SCP in all life stages but did not look at diapause, which is a key aspect of the survival strategy of an overwintering larva.

Two means of achieving cold tolerance were examined in this study: freeze tolerance via ice inoculation in both *C. vicina* and *C. vomitoria* and rapid cold hardening (RCH) in *C. vomitoria*. RCH has not been described in either species, but the RCH of *C. vicina* has recently been established within our laboratory (Coleman & Hayward, unpublished data). The determination

of the SCP can be made in one of two manners, in a dry medium or when the sample is placed in a moist surrounding and then an ice crystal is introduced when the alcohol bath is between -1 and -2^{o}C (termed ice inoculation). Freeze tolerance via ice inoculation has not previously been described in either *C. vicina* or *C. vomitoria*, although dry L3 larvae of *C. vomitoria* were established to be freeze intolerant (Block *et al*., 1988). Research by Kostal and Haverlka (2000) established that diapausing larvae of the gall midge, *Aphidoletes aphidmya,* were unable to survive freezing as dry larvae, but when inoculated with ice, larvae had a relatively high survival rate (23-34%). This highlights a further complication in an already dichotomic cold tolerance categorization method (Bale, 1993, 1996; Sinclair, 1999).

3.3. Method

3.3.1. Rearing of larvae and adults

All adults and larvae of *C.* vicina, *C. vomitoria* and their cross were reared according to the protocol stated in chapter 2.

3.3.2. SCP of C. vicina and C. vomitoria

The supercooling point (SCP) for each stage of the insect's life cycle was measured using PicoLog data acquisition software. Individual eggs/larvae/adults were attached to single K-type thermocouple probes, using a small amount of Oecotak™ grease (Oecos Ltd). The probes were connected to a multichannel thermocouple data logger (PicoTech TC-08), which relays information to a PC running PicoLog (example output is shown in fig. 3.1). Temperature was continuously recorded at 1 s intervals. Larvae and eggs were held individually within size three (1cm) Beem capsules (Agar Scientific Ltd., UK); adults were individually placed in an Eppendorf™ (1.5 mL) tube. Three to four Beem capsules/Eppendorfs™ were placed inside glass boiling tubes in a low temperature alcohol bath (Haake F8-C50, Thermo Haake, Germany). The temperature

was lowered from rearing temperature to -33°C at a rate of 0.5°C min^{-1}. The sample size was 16 for all life stages.

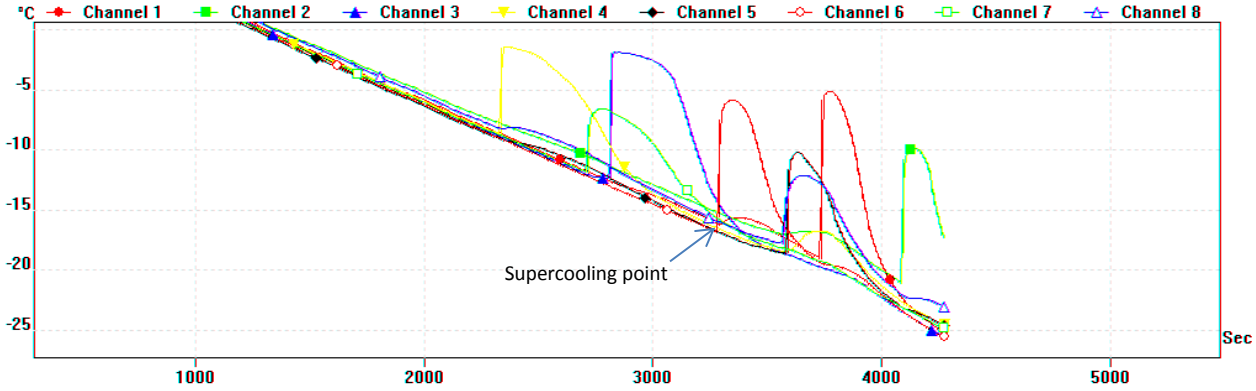


Figure 3.1. Example output of the exotherms of eight L3 non-diapause (ND) larvae of C. vicina. Readings were obtained using K-type thermocouples attached to a picotech TC-08 datalogger. Larvae were placed in Beem capsules, in contact with the thermocouple, and were cooled from 11°C to -33°C at 0.5°C min-1.

The data were illustrated as a continuous graph of decreasing temperature over time. The software recorded a peak (exotherm) in the temperature profile at the point at which each individual froze, the point where temperature begins to rise is usually referred to as the SCP.

Two photoperiods were established. A short day (SD) photoperiod was established in which a photoperiod of 12:12 (light:dark) was used and a long day (LD) photoperiod of 18:6 (light:dark) was also established. In *C. vicina*, the SCPs were recorded for SD (also termed non-diapause – ND; McWatters & Saunders, 1996) and LD (also termed diapause – D; McWatters & Saunders, 1996) larvae at the following ages post oviposition: 0 days (egg), 1d (L1), 2d (L2), 4d (L3 early, feeding stage) and 10d (L3 late, post wandering). The SCPs were also recorded for pupae (2 days post pupariation) and adults (newly emerged). For larvae that had entered diapause, the SCP was measured on day 0 of diapause (30 days post oviposition (Saunders, 1987) termed D0), then on D5, D10, D15, D20, D30, D40 and D50 of diapause.

In *C. vomitoria*, the SCPs of SD and LD individuals at egg, L1, L2, L3 early, L3 late, pupa and adult stages were recorded. The SCP of wet larvae was also established (see chapter 2 and 4 or

further information on the importance of moisture for *C. vomitoria*), as was the SCP of L3 larvae which had been acclimated at 5°C for 5 days prior to experimentation; this was due to larvae held at 11°C rapidly developing from L3 to pupariation (see chapter 2 for further details). In chapter 2 it was established that *C. vomitoria* do not enter diapause under thermal and photoperiodic conditions administered, as such they shall be referred to as LD or SD.

3.3.3. Assessment of Freeze Tolerance via ice inoculation

Freeze tolerance in L3 ND *C. vicina,* LD *C. vomitoria* and their cross was assessed by holding larvae on ice for 1 h before being placed in a large Beem capsule lined with moist tissue paper and inoculated with an ice crystal at c. -1.5°C. A small hole was made in the lid of the Beem capsule and a type K thermocouple inserted next to the larvae. The temperature probes were connected to a multichannel thermocouple data logger (see section 3.2.2 for more details). Initial studies were conducted to ensure that samples did not have multiple SCP's when cooled from 0°C to -33°C at a rate of 0.2°C min^{-1}, i.e. that the moist tissue and larvae did not have different SCPs. It was established that samples had just one super-cooling event and that all larvae had frozen by the time -5°C was reached. Subsequently, larvae were cooled from 0°C to -33°C using a ramping rate of 0.2°C min^{-1}. Larvae of *C. vicina* (n=16)*, C. vomitoria* (n=22) and their cross (n=17) were removed at their SCP (as indicated by an exotherm being recorded). In a second experiment, larvae of *C. vicina* (n=24) and *C. vomitoria* (n=70) were cooled from 0 to -5.5°C (-5.5°C was used as it was lower than the lowest recorded SCP during initial studies) using a ramping rate of 0.2°C min^{-1}; these larvae were then held for 1h at -5.5°C. After each treatment, larvae were warmed to 5°C at 0.2°C $min^{-1,}$ and then transferred to 11°C for 24h before being moved to 20°C until eclosion. Larvae of *C. vomitoria* were also exposed to longer durations at -5.5°C of 4 h (n=16) and 24 h (n=16) before being cooled and re-warmed at 0.2°C min^{-1}. Survival was recorded as percentage of samples pupariating, and then as percentage eclosion success (Saunders and Hayward, 1998).

3.3.4. RCH in C. vomitoria

To determine the discriminating temperature (DTemp) samples of 10 larvae were each placed in a boiling tube (N=8) and plunged into a temperature-controlled alcohol bath for 2 hours (Haake F8-C50, Thermo Haake, Germany), set at each of the 0.5^{o}C increments between 0 and -20^{o}C. The DTemp is determined as being the temperature at which approximately 20% survival is induced. The ability of ND L3 *C. vomitoria* to rapidly cold harden (RCH) was investigated using 4 different treatments: 1. Gradual cooling at 0.5^{o}C min^{-1} from culture temperature (11^{o}C) to their DTemp, where they remained for 2h before being gradually re-warmed at 0.5^{o}C min^{-1}. 2. Acclimation of larvae at 0^{o}C before being held at the DTemp for 2h. 3. Acclimation of larvae at 2^{o}C before being held at the DTemp for 2h. 4. Acclimation of larvae at 5^{o}C before being held at the DTemp for 2h. All larvae were progeny of adults held at LD photoperiod (18:6 light: dark) at 15^{o}C; larvae were held at 11^{o}C in constant darkness both prior to and post experiments. Survival was recorded as percentage of samples pupariating, and then as eclosion success.

3.3.5. Statistical analysis

All data was subjected to a levanes test for equal varience and a Kolmogorov-Smirnov test to test for equal distribution. Differences between the SCP values were assessed using the Students t-test (Predictive Analytics SoftWare, PASW statistics 18). A linear regression of the SCP of *C. vicina* larvae at different times through diapause was carried out using PASW (statistics 18) to determine whether SCP alters as diapause progresses. The interquartile range was used to assess the spread of data, as this avoided results being affected by outliers. The difference between survival rates of acclimated and non-acclimated (direct transfer) groups within RCH experiments was assessed using an ANOVA in PASW statistics, performed with a LSD *Post hoc* test. Differences in the survival rates of ice-inoculated *C. vicina* and *C. vomitoria* individuals which had been either taken to their SCP or held for 1h at -5.5^{o}C, were assessed using a Student's T-test (Excel).

3.4. Results

3.4.1. SCP of C. vomitoria and C. vicina

Over all life stages, *C. vicina* had a lower SCP than *C. vomitoria* (fig. 3.2). Using a pairwise comparison between LD and SD, significant differences were noted in pupae of *C. vicina* ($P<0.001$), L2 larvae of *C. vomitoria* ($P=0.005$), pupae of *C. vomitoria* ($P=0.04$) and adults of *C. vomitoria* ($P=0.049$) only. Note that for *C. vicina*, LD and SD imply ND and D, respectively (McWatters & Saunders, 1996); *C. vomitoria* does not enter diapause under tested conditions (chapter 1), so the D and SD definitions do not apply.

In both species, eggs had the lowest SCP value (fig. 3.2), with short day (SD) *C. vicina* having a mean SCP of -26.00°C (± 0.28) and *C. vomitoria* -25.20°C (± 0.14) (fig. 3.2.). After larvae had completed the egg stage, the SCP increased in the L1 stage (-22.13 ±1.58°C, *C. vicina;* -16.00 ± 1.61°C, *C. vomitoria*) and further increased in the L2 stage (-14.53 ± 1.08°C and -7.69 ± 0.61°C respectively) (fig. 3.2). In all but one experiment (LD *C. vomitoria*), the SCP increased until the early L3 stage. It was also noted that there was an increase in the interquartile range (table 3.1) of SCPs in the L1, L2 and L3-early stages for both *C. vicina* and *C. vomitoria*, when compared to the egg stage, suggesting that there is an increase in variability through these stages.

Table 3.1. The interquartile range of SCP in the first four life stages of *C. vicina*. Larvae/eggs are progeny of adults which have experienced either a short (12:12 Light:dark) or a long (18:6) photoperiod. Eggs were used on the day of mass oviposition, 'L1' larvae were 1 day, 'L2' 2 days and 'L3 early' 4 days old. Larvae/eggs were secured on to a thercouple with Oecotak™ inside a Beem capsule and cooled at 0.5°C min^{-1} until the SCP was reached. N=16 for all stages, photoperiods and species.

	C. vicina		*C. vomitoria*	
	Short day (Diapause destined) (°C)	Long Day (Non-diapause) (°C)	Short day (°C)	Long day (°C)
Egg	1.05	1.43	1.82	0.48
L1	1.50	7.40	11.05	9.30
L2	10.20	2.20	5.80	3.88
L3 early	5.80	2.95	1.27	0.84

A greater number of significant differences were noted when *C. vicina* and *C. vomitoria* were compared to each other. In LD samples, significant differences were noted between L1 ($P=0.001$), L2 ($P=0.001$), L3 late ($P<0.001$), pupae ($P<0.001$) and adults ($P=0.012$). In SD samples, significant differences were noted in L1 ($P<0.001$), L2 ($P<0.001$), L3 late ($P=0.03$) and pupae ($P=0.003$).

During diapause (D0 to D20), the SCP of *C. vicina* larvae significantly decreased from -16.57^{o}C at D0 to -21.41^{o}C at D20 ($P<0.001$). When L3-late larvae of *C. vomitoria* were acclimated for 5d at 5^{o}C, they had a mean SCP of -10.54^{o}C ($\pm$0.27). L3 larvae that had been allowed to pupariate in damp sawdust had a higher SCP than larvae which pupariated in dry sawdust (-9.56 $\pm$ 0.56^{o}C and -14.96 $\pm$ 0.89^{o}C, respectively). When taken below their SCP to -33^{o}C (frozen) under dry conditions, no life stages of either species survived.

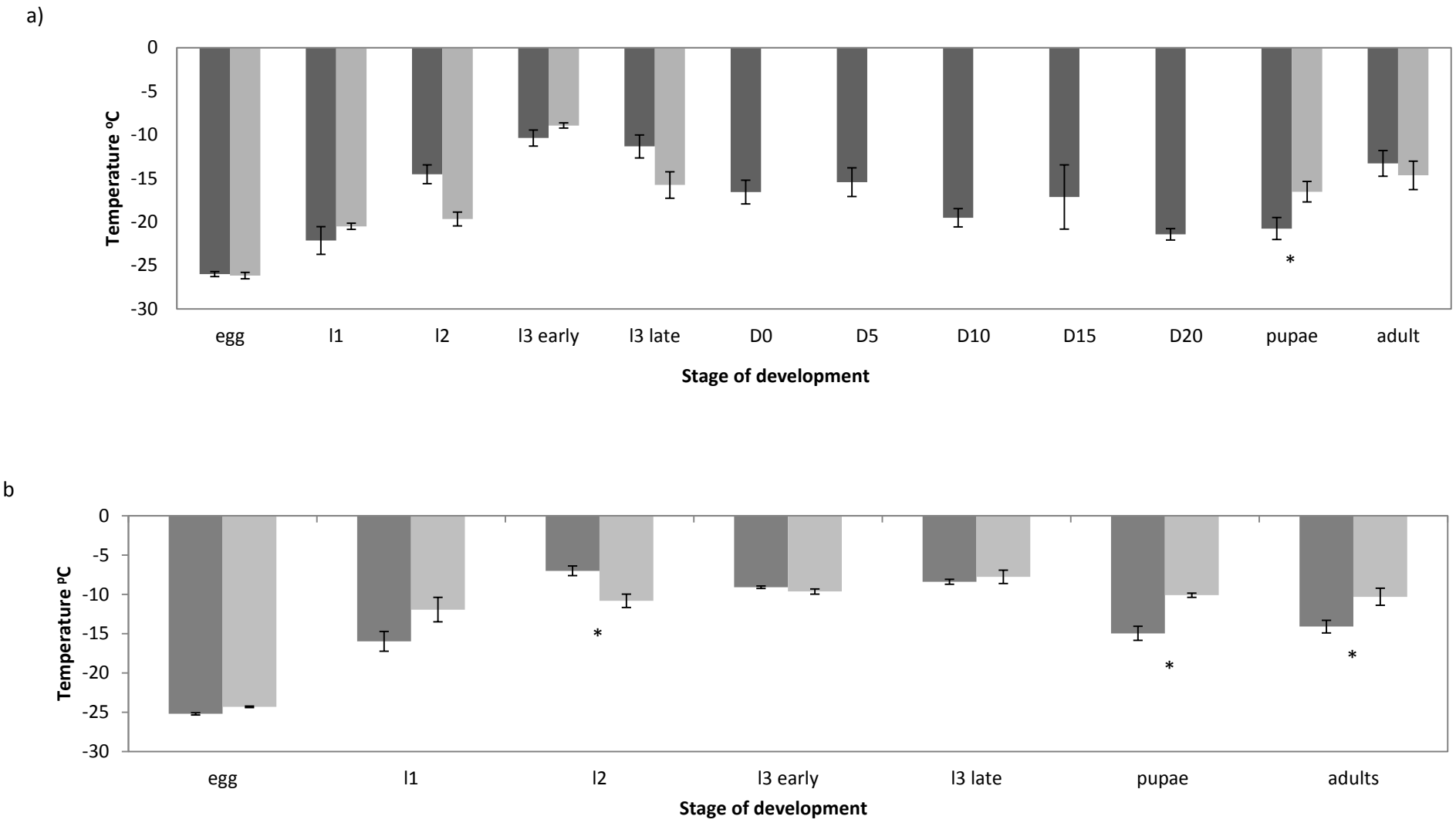


Figure 3.2. Mean SCP (±SE) of different developmental stages in the life cycle of a) *C. vicina* and b) *C. vomitoria*. Adults were reared under either long day (LD) (light grey) or short day (SD) conditions (dark grey), and the SCP of their progeny through to adulthood was assessed (n=16). Significant differnces between LD and SD are indicated with an asterisk.

3.4.2. Assessment of Freeze Tolerance

When larvae of *C. vicina* and *C. vomitoria* were cooled in a dry environment, their SCP was significantly different (P<0.001 in both cases), when compared to larvae cooled in a damp environment and inoculated with ice (fig. 3.3). No significant differences were seen between the SCPs of inoculated *C. vicina* and *C. vomitoria* (P=0.09). The SCP of the cross was significantly different to *C. vicina* but not to *C. vomitoria* (P=0.01 and P=0.32).

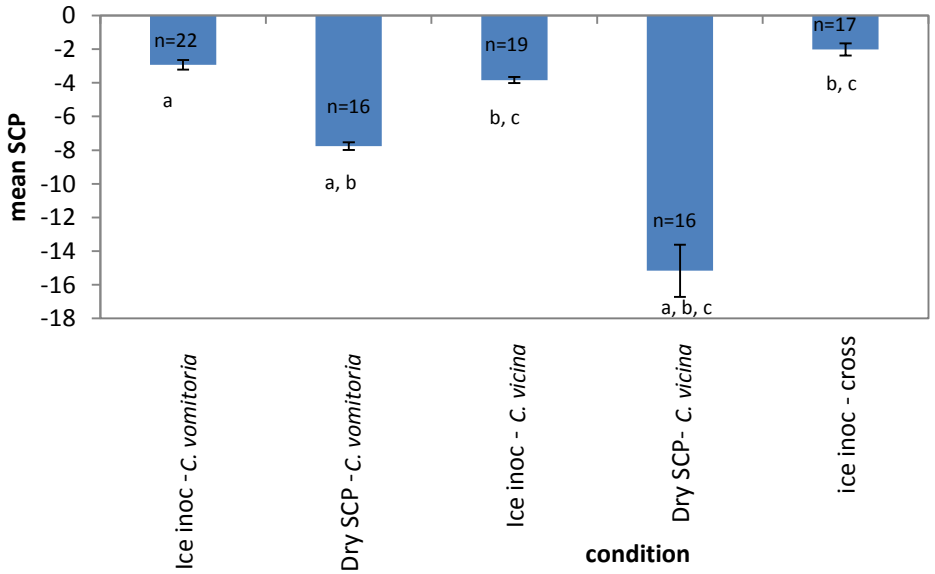


Figure 3.3. The mean SCP (±SE) of *C. vicina* and *C. vomitoria* when either inoculated with ice (termed 'Ice inoc') or held in dry conditions (termed Dry SCP). The SCPs of dry samples were determined by cooling at a rate of 0.5°C min^{-1}, the SCPs of ice inoculated samples were determined by cooling at 0.2°C min^{-1}. Groups that share the same letter are significantly different to each other.

Calliphora vicina and *C. vomitoria* appear to differ in their ability to tolerate inoculative freezing under wet conditions; 12.5% of *C. vicina* survived to pupariation following exposure to their SCP, but none survived to eclosion (fig. 3.4). No *C. vicina* tolerated 1h at -5.5°C (fig 3.4). In contrast, 49% of *C. vomitoria* survived to eclose successfully after exposure to their SCP as L3 larvae (fig 3.4) and 16% survived 1hr at -5.5°C (fig. 3.5). *Calliphora vomitoria* showed 0% survival when held at -5.5°C for either 4h or 16h (data not shown). The cross of *C. vicina* and *C. vomitoria* showed an intermediary response with 52.94% of larvae surviving to pupariation and 17.65% surviving to eclosion (fig. 3.4).

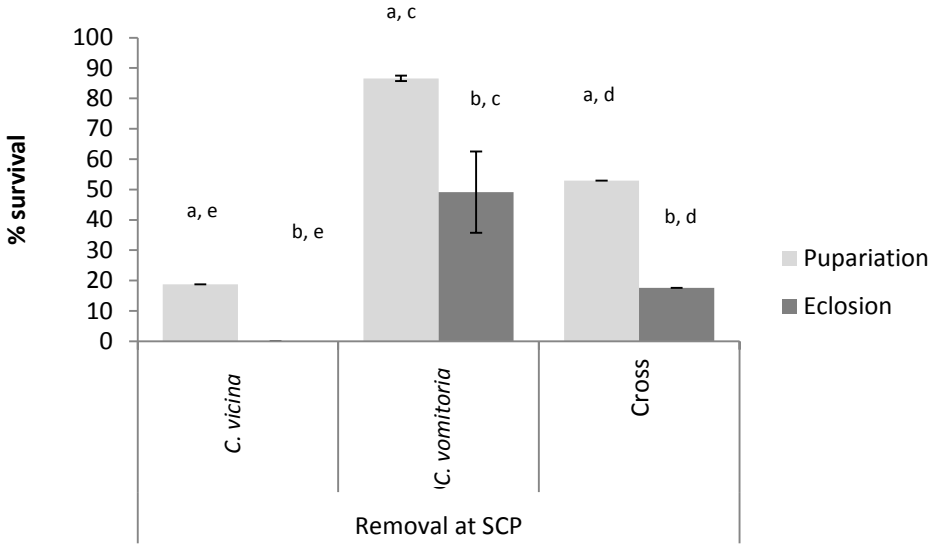


Figure 3.4. Survival to pupariation (light grey) and eclosion (dark grey) of *C. vicina* (n=16), *C. vomitoria* (n=22) and their cross (n=17) when inoculated with ice at c. -1.5°C during cooling from 5°C to -33°C at 0.2°C min^{-1}; after their SCP was reached, larvae were removed. The percentage pupariation and survival are represented as mean values (±SE). Groups that share the same letter are significantly different to each other.

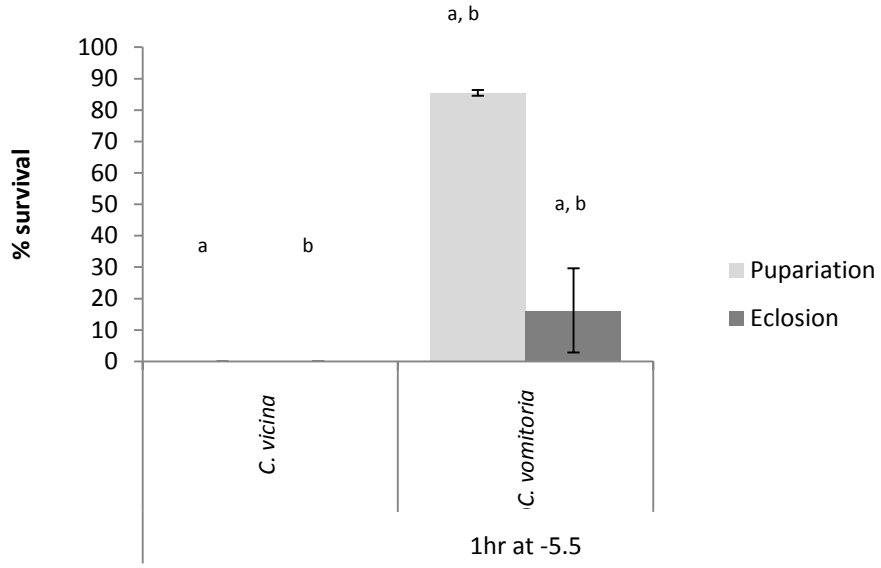


Figure 3.5. Survival to pupariation (light grey) and eclosion (dark grey) of *C. vicina* (n=24) and *C. vomitoria* (n=70), when inoculated with ice at c. -5.5°C during cooling from 5°C to -5.5°C at a rate of at 0.2°C min^{-1}. Larvae were then held at -5.5°C for 1h before being re-warmed at 0.2°C min^{-1}. The percentage surviving to pupariation and eclosion are represented as mean values (±SE). Groups that share the same letter are significantly different to each other.

3.4.3. RCH in C. vomitoria

The discriminating treatment for LD L3 *C. vomitoria* larvae was determined to be 2h at -13.0°C, after which 19.2% of larvae survived. All acclimation treatments resulted in increased survival at the discriminating treatment when compared to direct transfer (fig. 3.6.). Larvae that had been

acclimated for 2h at -5°C prior to the discriminating treatment showed 77.5% survival to eclosion, which was a significantly higher survival rate than for larvae that were directly transferred (P<0.001). Acclimation to -2°C for 2h resulted in 62.5% of larvae surviving and acclimation for 2h at 0°C resulted in 48.8% survival, both of which were significantly higher than direct transfer (P<0.001). Exposing larvae to a ramp of 0.5°C min^{-1} from 11°C to -13°C was the least successful of all acclimation treatments with 45% of larvae surviving to eclosion, although survival was still significantly higher than direct transfer alone (P<0.001). *Post hoc* test showed that all groups were significantly different (P<0.001) to each other with the exception of one pair: 2h acclimations at -2°C and -5°C.

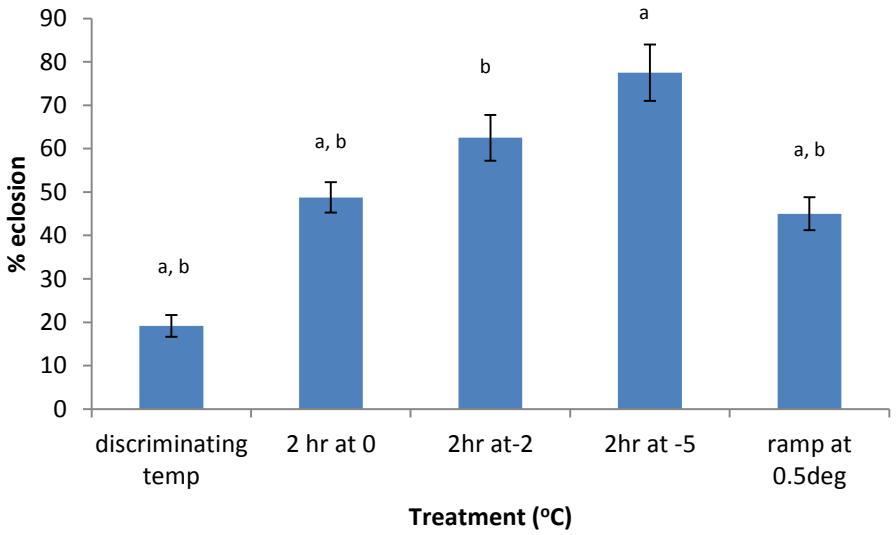


Figure 3.6. Mean percentage (±SE) of larvae surviving to eclosion after 2h at -13°C (the discriminating treatment). Four pre-treatments were established, a 2h acclimation at either 0°C, -2°C, -5°C or a ramp from 11°C to -13°C at 0.5°C min^{-1}, before a 2h exposure to the discriminating temperature. Groups that share the same letter are significantly different to each other.

3.5. Discussion

Cold tolerance is not a fixed trait within a species, but can be altered throughout winter; for instance, the concentration of cryoprotectants and levels of anti-freeze proteins have been seen to change during winter (Baust and Nishino, 1991; Meier & Zettel, 1997). The SCP has also been seen to alter as a consequence of experiencing a freeze thaw cycle in both the Antarctic beetle,

Hydromedion sparsutum (Bale *et al.*, 2001) and the hoverfly, *S. ribesii* (Brown *et al*., 2004). The presence of environmental water is one such climatic condition that is often overlooked, with the majority of studies examining cold tolerance of dry specimens. In this study, we found moisture to dramatically affect the SCP and therefore cold tolerance. When *C. vomitoria* pupae were continuously held for >2d in damp sawdust, their SCP was 5^{o}C higher than when held in dry sawdust. Furthermore, moisture had a large effect on L3 larvae of both *C. vicina* and *C. vomitoria,* with regard to both SCP and subsequent survival of dry vs. ice-inoculated larvae. The change in SCP for both *C. vicina* and *C. vomitoria* was dramatic, with *C. vicina* experiencing an 11.3^{o}C increase when larvae were inoculated with ice, rather than frozen whilst dry; in *C. vomitoria* there was a 4.8^{o}C increase (fig. 3.3). In this study, *C. vicina* were found to be freeze intolerant with 0% of larvae surviving to eclosion when removed at their SCP, after being frozen via ice inoculation. *Calliphora vomitoria*, however, showed an ability to tolerate freezing under wet conditions, with 49.1% surviving to eclosion after being removed at their SCP in the L3 stage. After being held frozen at -5.5^{o}C for 1h, survival decreased to just 16.2%. Research by Block *et al.* (1988) on *C. vicina* and *C. vomitoria* concluded that both species were freeze intolerant, with *C. vicina* being unable to tolerate any level of ice formation and *C. vomitoria* tolerating a very limited amount of internal ice formation, dependent on the site of formation and duration of the presence of internal ice. 50% (n=3) of post-feeding larvae survived to pupariation when larvae were removed soon after they had supercooled, but this quickly declined to 0% when the temperature decreased much below their SCP (Block *et al*., 1988). The difference in SCP between these two studies can be explained by ice being a very effective agent for inducing freezing within insects (Hart & Bale, 1998; Denlinger & Lee, 2010). As insects that are ice-inoculated have a higher freezing temperature, the ice crystals that form within are able to develop slowly, allowing cryoprotectants to be mobilised as ice is formed (Lee & Costanzo, 1998; Holmstrup *et al.,* 1999). One example of this is seen in the centipede, *Lithiobius forficatus*, in which cooling to its SCP (-3^{o}C) results in mortality, but when inoculated with ice at -1^{o}C it can

survive to temperatures as low as -6°C (Tursman *et al*., 1994); this effect was also seen in the Dipteran gall fly, *Eurosta solidaginis* (Lee & Hankinson, 2003). The ability of *C. vomitoria* to survive only a limited amount of internal ice formation previously led to the species being classified as freeze intolerant. Hawes *et al.* (2010) disputed this when cold tolerance experiments on the New Zealand Magpie Moth, *Nyctemera annulata*, revealed a similar 'partial freeze tolerance' strategy, with 27% surviving freezing when removed at the SCP. In a separate study, three species of *Tipula* (Diptera) also showed 'partial freeze tolerance' (43-68% survival) when they were removed at their SCP (Brown *et al.,* 2004). This adds a further dimension to the classification system of cold hardiness strategies; currently an insect's cold hardiness classification is based solely on experiments under dry conditions, despite many temperate insects being exposed to environmental water and/or ice during winter.

The first study to examine the SCP of all life stages, though not diapause, of both *C. vicina* and *C. vomitoria* was conducted by Block *et al.* (1990) in which it was concluded that eggs had the lowest SCP, after which SCP would generally increase through L1, L2 and L3-early stages, which is in agreement with this study. One notable difference is that in all but two life stages, the SCP was seen to be higher (c. 3°C) in research by Block *et al.* (1990), than found in this study. This could be due to one of three reasons: firstly, adults of the maternal generation were raised at the higher temperature of 25°C in the study by Block *et al.* (1990) and it has been seen in this study that an increased temperature at which the maternal generation is reared can have a negative influence on the cold resistance of larval progeny (see chapter 5). Secondly, the temperature at which larvae are reared alters the SCP; in this study, L3-late *C. vomitoria* had a SCP 3°C lower when reared at 5°C, compared to when larvae were continuously held at 11°C. This pattern was also seen as a result of altering the rearing temperature of diapause larvae in the firebug, *Pyrrhocoris apterus* (Hodkova & Hodek, 1994). Lastly, the rate at which larvae are cooled was not identical between the studies; with Block *et al.* (1988) cooling larvae twice as quickly as in this study (1°C min^{-1}, compared to 0.5°C min^{-1} respectively). The rate of cooling can

modify both survival rate (Miller, 1978), and SCP (Salt, 1966) with larvae cooled at a slower rate freezing at a lower temperature (Ramløv & Westh, 1992). Both *C. vicina* and *C. vomitoria* are holometabolous insects. As such they undergo complete metamorphosis to reach adulthood. During the larval stages holometabolous insects store energy and nutrients in their fat body for metamorphosis. Therefore, their metabolome can change quite drastically, this along with body size can affect the temperature at which freezing occurs (Ramløv & Westh, 1993).

When the SCP is recorded for the duration of diapause (D0 to D20), a decrease in the SCP can be seen. In *C. vicina*, this decrease in SCP appears to be carried through to the pupae, which have a significantly lower SCP than non-diapause pupae. This decrease in SCP as diapause progresses is also seen in the codling moth, *Cydia pomonella*, where a 6^{o}C decrease in the SCP was observed as diapause progressed (Khani & Moharramipour, 2010). However, this trend is not universal; with H*yphantria cunea* raising its SCP over the period from November to April from -22.9^{o}C to -20.2^{o}C (Li *et al.,* 2001). This may be explained by progression through diapause, which is thought to lead to changes in cold tolerance and SCP (Goto *et al*., 2001), though the link between SCP and cold hardiness is disputed, as is that between cold tolerance and diapause (Tanaka, 1997).

Both the ecological significance and the experimental procedure of SCP experiments has recently been disputed. Wilson *et al*. (2003) argued that 200-300 measurements on a single sample was needed to get accurate results, they found that there was a 2^{o}C range in temperature on the SCP recorded from a single sample. This is obviously not possible on live organism, as freeze avoiding insects die once their SCP has been reached and freeze tolerant organisms would die due to accumulated freeze-thaw injuries before the number of repetitions required was reached. Renault *et al*. (2002) noted that their SCP results were bimodal due to sexual dimorphism, as such there are inherent errors if the sex is not recorded. This could explain some of the variability seen in this data (though a distinct bimodal distribution was not seen in any groups), as maggots are not sexually dimorphic the sexual identity of each maggot

would have to be ascertained through genetic experiments to assess if the SCP of either *C. vicina* or *C. vomitoria* differed based on sex. Renault *et al.* (2003) also questioned the ecological significance of the SCP being as though the SCP of tropical insects can be lower than temperate or sub-arctic insects, despite tropical species never experiencing cold temperatures; instead it is argued that the SCP may have more to do with desiccation resistance than cold resistance.

RCH is a widespread method of cold protection in insects (Kim & Kim, 1997; Rinehart *et al.,* 2000; Wang & Kang, 2005; Jensen *et al.,* 2007; Shintani & Ishikawa, 2007). This is the first study to present the RCH results of any Calliphoridae species and the only to present a comparison between two closely-related species that exhibit a differing cold tolerance strategy. The strongest RCH response in *C. vomitoria* was after acclimation to -5^{o}C for 2h, where survival increased from 19.2% to 77.5%. This is higher than the 66% increase seen in *C. vicina* under the same acclimation conditions (Coleman, unpublished data). Conversely, a ramping rate of 0.5^{o}C min^{-1} elicited the weakest RCH response in *C. vomitoria* (45%, fig. 3.4), but a strong response in *C. vicina* (>70%, Coleman, unpublished data).

This study has identified distinct differences in the influence of environmental moisture on the cold stress physiology and tolerance of these two closely-related species, as well as more subtle differences in their RCH response. As always, results should be examined in the light of the natural habitat in which the species resides. *Calliphora vicina* is synanthropic (Hwang and Turner, 2005) and therefore commonly overwinters in urban areas, therefore it is able to seek warmer or more sheltered microhabitat conditions, which might be less likely to induce inoculative freezing. In contrast, *C. vomitoria* are more associated with rural habitats (Erzincliogl u, 1996) and those at increased altitudes (Baz *et al.,* 2007) which may lead to rapid diurnal fluctuations in temperature. As such, *C. vomitoria* is potentially more likely to overwinter in wet conditions as well as being more likely to experience lower temperatures than *C. vicina*. A notable difference between the two species is that *C. vomitoria* are not thought to overwinter in

diapause (Block *et al*., 1988; chapter 2), thus any mechanisms which enable diapausing insects to increase winter survival would not be employed. Overwinter survival success in the monarch butterfly, *Danaus plexippus*, relies on its capacity to rapidly cold harden (Larsen, 1994), which may be the mechanism utilised in overwintering *C. vomitoria*. Unlike the monarch butterfly, larvae of *C. vomitoria* can survive a limited amount of internal freezing arising from inoculative freezing, but not for any extended lengths of time (e.g. ≥2h at -5.5^{o}C). As *C. vomitoria* are found at higher altitudes than *C. vicina* (Baz *et al.,* 2007), which experience greater diurnal temperature fluctuations, this may account for their greater capacity to rapidly cold harden. Environmental water appears to be an important factor that needs to be considered during the classification of cold tolerance. Both *C. vicina* and *C. vomitoria* showed an improved survival rate when ice inoculated. With *C. vicina*, there was an increase in survival to pupariation when ice inoculated samples were removed directly after freezing (17.6%) but none survived to eclosion; by contrast, in *C. vomitoria* a modest number of larvae survived freezing for 1 h and subsequently developed through to eclosion (16%). Results of this study suggest that *C. vomitoria* should be re-classified as 'partially' freeze tolerant. It is proposed that when classifying an organism's cold tolerance, moisture should not be excluded as a factor, especially in species that would encounter environmental moisture in their normal overwintering habitat.

It was noted in chapter 2 that *C. vomitoria* require damp media to enable a high (>90%) survival rate. Whereas *C. vicina* do not require such treatment. Furthermore, *C. vomitoria* were seen to have a large difference in their SCP if held in dry compared to damp sawdust. These together with the heightened survival rates in *C. vomitoria* if ice inoculated suggest that *C. vicina* and *C. vomitoria* may utalise water differently, as such, a comparison of desiccation in *C. vicina, C. vomitoria* and their cross would be very interesting.

4. Differences in the resistance and tolerance of desiccation and cross tolerance to cold in two species of Calliphoridae

4.1. Summary

Blow flies are highly cosmopolitan and species such as *C. vicina* are found throughout temperate and subarctic regions of the northern hemisphere, as well as tropical regions and even colonised sub-Antarctic islands in recent times (Lebouvier *et al*., 2011). When blowfly third instar (L3) larvae bury to a depth of 5-30cm in the soil they may be exposed to a broad range of moisture conditions and potential desiccation stress. This study compares the desiccation resistance and tolerance of two closely-related flies in the family Calliphoridae: *C. vicina* and *C. vomitoria.* Distinct differences in rates of water loss at 5% relative humidity (RH) were recorded; after 5d of desiccation *C. vicina* had lost just 17.2% of body water whereas *C. vomitoria* and the cross had lost 49.6% and 52.2%, respectively. There was also a distinct difference in water loss depending on whether larvae were desiccated as individuals or in groups. When groups of *C. vicina* were desiccated for 5d, *C. vicina* had lost 16% of initial water content and *C. vomitoria* had lost 23.1%. Tolerance of water loss data indicated that the cross was the most tolerant to desiccation, with a 20% mortality corresponding to a 33.9% loss of initial water content. *Calliphora vomitoria* had intermediate desiccation tolerance, exhibiting 20% mortality after 2.7d, by which time they had lost 23.4% of initial water content. *Calliphora vicina* were least tolerant of desiccation with 20% mortality after just 19.7% loss of initial water content (after 9.2d). Desiccation prior to cold exposure significantly enhanced cold tolerance, with *C. vicina* and *C. vomitoria* having a significantly higher eclosion success than non-desiccated controls when exposed to sub-zero temperatures (*C. vicina* were held at -9.5^{o}C for 2hrs, *C. vomitoria* at -13^{o}C for 2hrs) alone ($P=0.013$ and $P<0.001$ respectively). The ubiquitous nature of *C. vicina* may be enabled by its relatively high resistance to water loss; in the field larvae bury in soil for part of their life cycle and may remain there for many months, therefore resistance to desiccation will allow *C. vicina*

to survive in a greater range of microhabitats. *Calliphora vomitoria* appears to be desiccation sensitive and as such may only be able to survive in soils which are moist and not prone to drying out; this may limit the range of *C. vomitoria* and reveal one of the reasons why the species is not as ubiquitously distributed as *C. vicina*.

4.2. Introduction

Abiotic factors often have significant effects on the development (Ames & Turner, 2003), dormancy (Vinogradova & Zinovjev, 1972) and survival of insects (Nath *et al*., 1997). Water availability in particular is central to all life on earth, and can be a real challenge in dormant overwintering insects, due to the loss of water exchange methods, notably drinking/feeding (Hahn & Denlinger, 2007), excretion, the ability to travel to more favourable areas (Burges, 1960), and the inaccessibility of environmental water in frozen environments. Limited access to water can occur during any season however, and different developmental stages within an insect life cycle are known to have very different abilities to tolerate drought. For example, in a study by Hull-Saunders *et al.* (2003), all life stages of the golden tortoise beetle, *Charidotella bicolor,* were desiccated at <2% RH for 12h. All life stages differed in their ability to resist water loss with the most resistant stage being pupae and the least resistant being first instar larvae, a c. 90% difference in the percentage of total body water lost was seen between these two stages. Not only does desiccation differ between stages, but it also can change within a stage. Kawano *et al*. (2010) identified a gene, *Desiccate,* that was selectively up-regulated when holometabolous larvae of *D. melanogaster* were placed in dry conditions, but expression was reversed when transferred to wet environments; using RNAi overexpression of *Desiccate* was seen to increase desiccation resistance in L2 larvae. Some species of insect are highly desiccation resistant, with the goldenrod gall fly, *Eurosta solidaginis*, (Ramløv & Lee, 2000) and the desert beetle, *Onymacris plana*, (Hadley, 1994) reported to be amongst the most

desiccation resistant species. This heightened desiccation resistance is often enabled by impermeable cuticles that greatly restrict water loss, as was seen in *E. solidaginis* (Ramlov & Lee, 2000) and *O. plana* (Edney, 1971). Cuticular permeability can be decreased through the alteration of cuticular hydrocarbons by increasing the compactness, linearity and length and saturation (Benoit, 2010). A decrease in the permeability of the cuticle leads to evaporative water loss occurring predominantly through the respiratory spiracles (Zachariassen & Maloiy, 1989). Other species may be vulnerable to water loss even at very high humidities, for example the collembolan *Folsomia candida,* lost a third of its initial total water content after just 24h at 98.2%RH (Bayley & Holmstrup, 1999). However, limited desiccation does not necessarily mean limited tolerance, and there are many species that lose water very easily but are extremely resistant to desiccation. For example, larvae of the sleeping chironomid, *Polypedilum vanderplanki,* when desiccated at 5%RH will lose almost 100% of its water content within 48h, but after just 1h in water will rehydrate with good survival (Cornette & Kikawada, 2011).

Roughly 70% of an animal's body weight is water, and variation in cellular water can lead to disruptions in cellular function due to changes of ionic concentrations, steric organelle organisation and their subsequent interactions with intracellular enzymes and proteins (Denlinger & Lee, 2010). One of the central mechanisms employed by organisms undergoing desiccation is an increase in polyols and sugars. For example, glycerol is up-regulated in response to desiccation in the beetle *Cucujus clavipes,* (Bennett *et al.*, 2005), trehalose was up-regulated in the Antarctic nematode *Plectus murrayi,* (Adhikari *et al.,* 2010) and *P. vanderplanki* (Cornette & Kikwada, 2011), sorbitol, glucose, alanine, betaine and mannitol were all seen to increase in the earthworm *Dendrobaena octaedra* (Pedersen *et al.,* 2008). Polyols and sugars are known to alter cellular osmolality (Bayley & Holmstrup, 1999), lower the membrane phase transition temperature (Crowe & Crowe, 1986) and increase supercooling capacity (Bayley & Holmstrup, 1999). They also inhibit protein denaturation (Crowe *et al.,* 1992) and stabilise cell contents by creating an intracellular glass state (Crowe *et al*., 1998). Heat-shock proteins have

also been shown to be up-regulated during desiccation, for example in the flesh fly, *Sarcophaga crassipalpis,* non-diapause pupae saw an up-regulation in the expression of HSP23 and HSP70 due to desiccation at <5%RH (Hayward *et al.,* 2004).

Survival rates of desiccation can be improved by acclimation to a less severe desiccation and a reduced desiccation rate; for example, in the springtail, *Folsomia candida,* acute desiccation for 6d at 95%RH was seen to result in 0% of desiccated pupae surviving to eclosion, but when acclimated at 98.2%RH for 6d before the more severe desiccation, survival rate increased to c. 65% (Sjursen *et al.,* 2001). One explanation for the higher survival rate when experiencing a slower rate of desiccation is that more time is allowed for the increased production of osmolytes; for example, in *D. octaedra* sorbitol concentration increased from 0.001mol kg^{-1} in control samples to 1.539mol kg^{-1} after 14d of gradual desiccation, acute desiccation for 4d led to a sorbitol concentration of 0.037mol kg^{-1} (Pedersen *et al*, 2008).

One means that insects use to reduce the rate of water loss is aggregation, thus decreasing the surface area of each insect exposed to low RH conditions, and also creating a less stressful microclimate by raising local RH. Evidence of reduced water loss in groups is provided by Benoit *et al*. (2007a) who recorded that there was a strong positive relationship between suppression of water loss and aggregation size in the bed bug, *Cimex lectularius.* A decrease in water loss was also recorded in the collembolan *Cryptopygus antarcticus,* which decreased from c. 11.5% water loss per hour when held individually to c. 6%/h when held in groups of 500 (Benoit *et al*., 2008). An increase in aggregation as humidity decreased was seen in the Antarctic midge, *Belgica antarctica* (Benoit *et al*., 2007b).

Desiccation would have been the primary stress that organisms initially faced when first adapting to life on land. It has been proposed that cold tolerance mechanisms evolved from those employed in response to desiccation, and there are certainly many similarities (Block, 1996; Ring & Danks, 1994). For example, the polyol glycerol is thought to be up-regulated in

response to low temperatures and desiccation (Yoder *et al.,* 2006) and the sugar trehalose is up-regulated both in response to cold shock (Overgaard *et al.,* 2007) and desiccation (Benoit *et al*., 2009). Heat-shock protein expression is also increased due to both exposure to low temperatures (Rinehart *et al*., 2007) and desiccation (Gusev *et al*., 2011). As the mechanisms are evolutionarily closely-related, it is thought that in some species a cross tolerance of desiccation and cold tolerance can be displayed. *Belgica antarctica* is one organism that displays this cross tolerance phenotype; control samples which experienced no desiccation prior to a cold stress (48h at -10^{o}C) showed low survival rates of c.10%, but when *B. antarctica* were exposed to a pre-treatment of 48h at 98.2% RH, survival increased to 100% (Hayward *et al*., 2007).

This study was conducted in order to gain further insights into the desiccation response found in the holometabolomic insects i*C. vicina*, *C. vomitoria* and their hybrid cross. *Calliphora vicina* is a freeze-intolerant species (Block *et al.*, 1988; Chapter 3), inhabiting the majority of the northern temperate region (Stevens & Wall, 2001; Fischer *et al*., 2004; Hwang and Turner, 2005; Liu *et al*., 2011), through to the sub-Antarctic (Lebouvier *et al*., 2011). *Calliphora vomitoria* is thought to be 'partially' freeze tolerant (chapter 3); its range appears to be confined to the Northern Hemisphere, being recorded in North America (Brundage *et al*., 2011) and from Western Europe (Hwang and Turner, 2005; Niederegger *et al*., 2010) to Japan (Iwasa & Inoue 2012). To our knowledge, the desiccation tolerance of either species has not been previously investigated. In this study, experiments were conducted to establish four key objectives: elucidate the desiccation resistance of *C. vicina, C. vomitoria* and their cross, determine whether desiccation rate differed between *C. vicina* and *C. vomitoria* when larvae were either individually desiccated or desiccated in groups, compare the desiccation tolerance of both species and their cross, and finally, to assess whether *C. vicina* and *C. vomitoria* exhibit an enhanced cold tolerance when partially desiccated.

4.3. Method

4.3.1. Larval cultures

Each species was cultured as described in chapter 2. The hybrid (cross) was created from adult female *C. vomitoria* and male *C. vicina*; the reciprocal cross was not viable. Non-diapause (ND) larvae were established by exposing the maternal adult generation to an 18:6 L:D photoperiod at 20°C. Larvae were then held at 11°C in constant darkness. Post-wandering ND larvae were used in experiments two days post gut evacuation.

4.3.2. Desiccation resistance

To compare the desiccation resistance of *C. vicina*, *C. vomitoria* and the cross, a relative humidity (RH) of <5% was set up using Drierite™ (calcium sulphate [$CaSO_4$]) inside an airtight desiccation chamber. The <5%RH was confirmed using a hygrometer (Hygrochron™ iButton DS1923, Maxim Integrated). Individual post-wandering L3 larvae were housed inside open 5ml microcentrifuge tubes placed within the desiccation chamber (fig. 4.1). Following Raoult's law, the air inside the closed system quickly equilibrated.

Eight replicates of individual ND *C. vicina*, *C. vomitoria* and cross larvae were weighed to record their fresh / starting mass (*s*) and then held at <5%RH/ 5°C for 21 days (d). At the same time each day, all larvae were removed and weighed to record their desiccated mass (*f*), and then returned to the desiccation chamber. On completion of the desiccation experiment, samples were dried to a constant mass at 60°C over 2d, and their dry mass recorded (*d*). From these values, starting water content and daily water loss were calculated using the following formulae:

Initial water content (*i*) = *s*-*d*.

Percentage of initial water remaining (calculated for each day)= 100 (*f*-*d*/i)

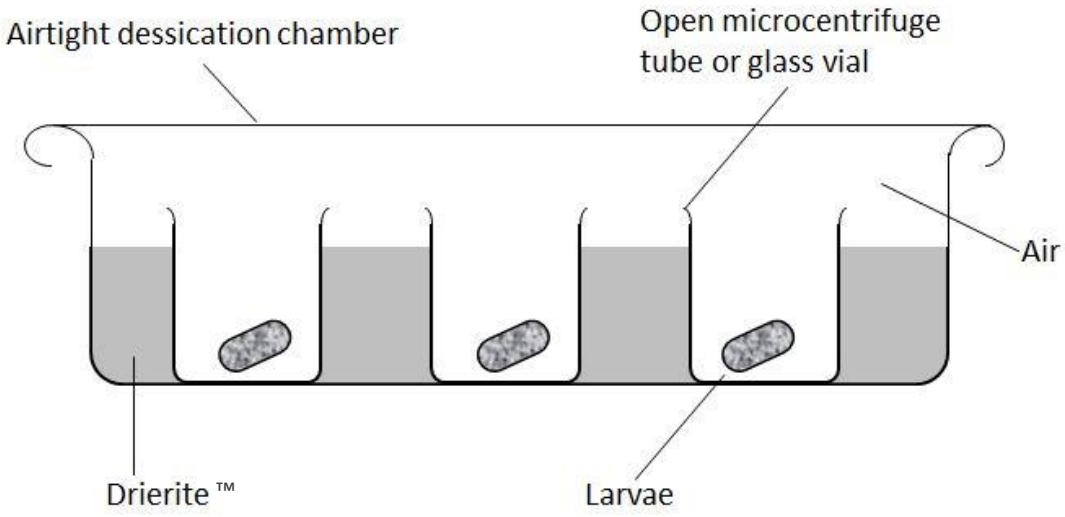


Figure 4.1. Airtight desiccation chamber, containing an open microcentrifuge tubes or glass vials which are surrounded to the shoulders by Drierite™ and sealed within an airtight container.

To test the hypothesis that larvae group size had an effect on the rate of water loss in *C. vicina* or *C. vomitoria,* groups of 10 L3 ND larvae were placed in glass vials (eight reps. per species) at <5% RH/ 5^{o}C, and water loss rates calculated as described earlier. These results were compared with those for individual larvae.

To account for the moist air entering the system when larvae were removed daily a large excess of Drierite™ was used, both during test runs and the actual experiments humidity inside the container was recorded using a hygrometer and the air inside the container quickly equilibrated after larvae were weighed each day, no change in the amount of time to equilibrate was noted as the experiment progressed.

4.3.3. Desiccation tolerance

Individual ND larvae of *C. vicina*, *C. vomitoria* and their cross were desiccated at <5% RH/ 5^{o}C, as described above. Eight larvae of each species were removed each day for 14d. Survival was

assessed as the percentage of larvae that pupariated and eclosed following each desiccation exposure.

To test the hypothesis that desiccation tolerance was greater in larvae that were desiccated in groups (due to reduced rates of water loss – see section 4.3.2. on groups), groups of N=10 larvae were exposed to <5%RH/ 5^{o}C and survival assessed daily, as described above. Three replicates (n=10) were removed daily for 7d and the percentage that had pupariated and eclosed was recorded.

4.3.4. Cross tolerance to cold

To determine whether desiccation enhanced the cold tolerance in *C. vicina* and *C. vomitoria,* eight replicate glass vials, each containing eight larvae, were desiccated at <5% RH/ 5^{o}C for either 24h (*C. vomitoria*) or 4d (*C. vicina*). These exposures were selected because they resulted in approximately 10% water loss (fig. 4.2), but had no significant effect on survival (fig. 4.4). Following desiccation, larvae were transferred to a boiling tube containing sawdust and placed in a temperature controlled alcohol bath. *Calliphora vomitoria* larvae were held at -13^{o}C for 2h and *C. vicina* larvae were held at -9.5^{o}C for 2h. These conditions represented the DTemp for the respective species (non-acclimated - see chapter 3). After exposure to sub-zero temperatures, larvae were held at 11^{o}C for 24h before being transferred to 20^{o}C until eclosion. Control samples were held in glass vials, each containing eight larvae (N=8) and were held at 5^{o}C for 24h (*C. vomitoria*) or 4d (*C. vicina*), before undergoing the stress and recovery treatments described above. In all experiments, after being removed from sub-zero temperatures, *C. vicina* were held in a petri-dish containing dry sawdust and *C. vomitoria* in damp sawdust (1:1, weight of water:sawdust – see chapter 2).

4.3.5. Statistical analysis

Time to a predetermined mortality level (20%) under desiccation treatment was assessed using a probit analysis. An ANCOVA was used to analyse the water loss in the different species for both individuals and groups. To determine whether desiccation significantly enhanced the cold tolerance of *C. vicina* and *C. vomitoria* larvae relative to un-desiccated controls, a Kruskal-Wallis test was performed, and *post hoc* Mann-Whitney tests were performed to compare amongst the different treatments. All statistical tests were completed using PASW (Predictive Analytics SoftWare, PASW statistics, v.18.0.0).

4.4. Results

4.4.1. Desiccation resistance

Calliphora vicina lost water at the slowest rate at <5%RH, while *C. vomitoria* and the cross had the a lower resistance to desiccation (fig. 4.2). The rate of water loss was significantly different between *C. vicina* and *C. vomitoria* (ANCOVA, $F=82.181$, $P<0.001$, Partial Eta Squared = 0.804) and between *C. vicina* and the cross (ANCOVA, $F=58.875$, $P<0.001$, Partial Eta Squared = 0.808). Water loss rates were not significantly different between *C. vomitoria* and the cross (ANCOVA, $F=4.758$, $P=0.057$, Partial Eta Squared = 0.154). The hybrid larvae were held for 15d at <5% RH; after 10d larvae became unresponsive to mechanical stimulus and the percentage water loss plateaued, therefore larvae were removed after 15d and oven dried to a constant mass.

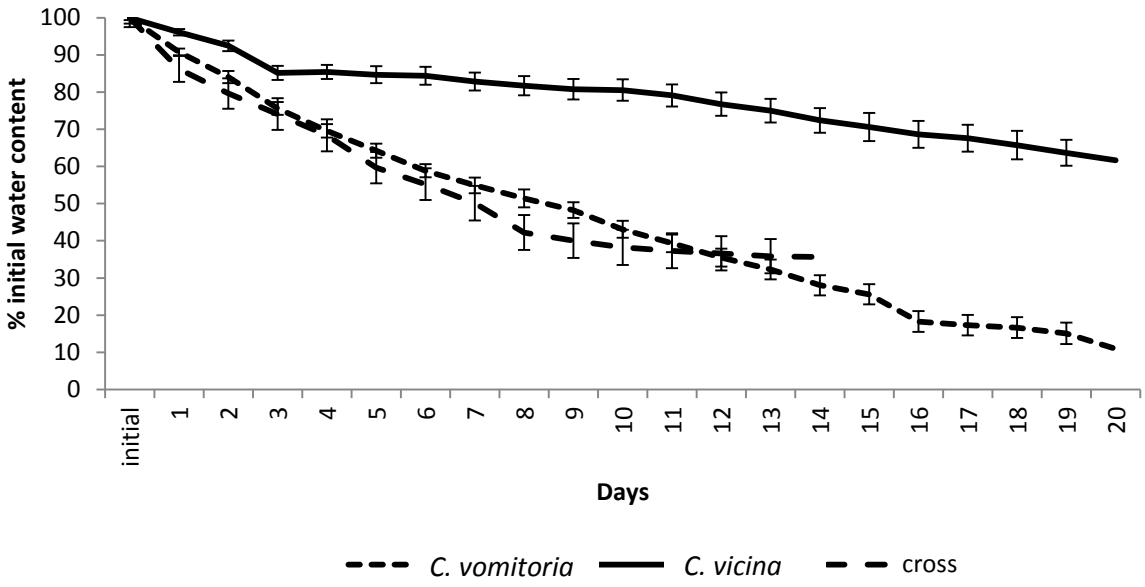


Figure 4.2 Mean percentage water loss (±SE) of individual L3 larvae of *C. vicina, C .vomitoria* and their cross, following different durations of exposure to <5% RH at 5°C. N=8 for all experiments.

4.4.1.1. Effect of group size

When comparing the effect of water loss on individuals compared to groups, both *C. vicina* (fig. 4.3a) and *C. vomitoria* (fig. 4.3b) showed a significantly slower rate of water loss over 14d when held in groups, compared to when larvae were held individually ($F= 31.972$, $P<0.001$, Partial Eta Squared = 0.711 and $F= 37.204$, $P<0.001$, Partial Eta Squared = 0.741 respectively). After 10 days at <5%RH, individual larvae of *C. vicina* had 64.1% of their initial water content remaining, whereas groups of *C. vicina* had 74.8% of initial water content remaining (fig. 4.3a). A similar, but more pronounced, pattern was seen in *C. vomitoria* with individuals losing a higher percentage of water content; groups of larvae retained 53.9% whereas individual larvae retained just 20.0% of water content after 10 days (fig. 4.3b).

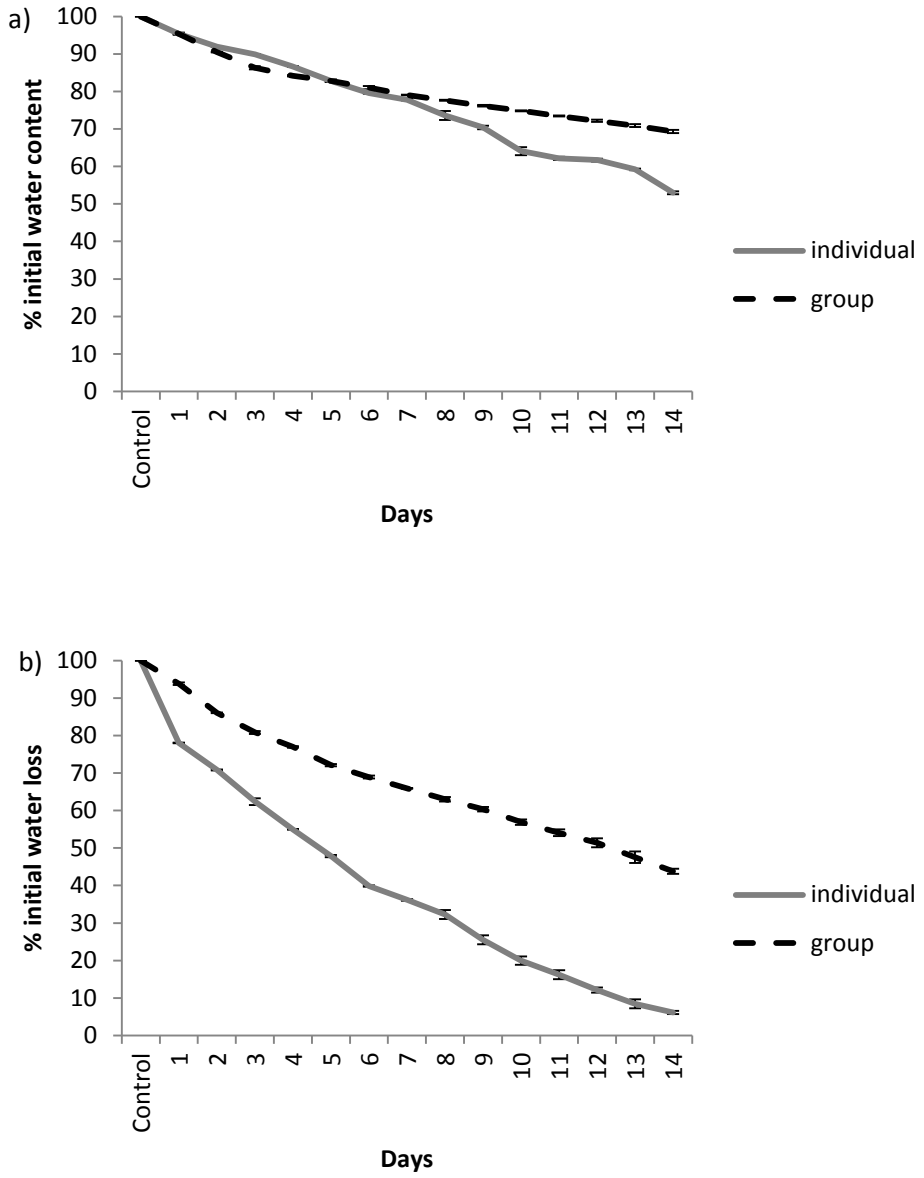


Figure 4.3 Mean percentage water loss (±SE) of L3 larvae of *C. vicina* (a) and *C. vomitoria* (b) following different durations of exposure to low humidity. Larvae were either held in groups of ten larvae and placed in glass vials (N=8), or were individually held in 5ml microcentrifuge tubes (N=8). All larvae were surrounded by Drierite™ at 5°C/ <5%RH.

4.4.1.2. Desiccation tolerance

Although individually desiccated *C. vicina* showed a greater survival rate than either *C. vomitoria* or the cross when held in <5%RH (fig. 4.4), when desiccation tolerance was calculated, they were not the most tolerant. Tolerance to desiccation (<5%RH) was assessed by observing the water loss that occurred once the larvae had experienced a predetermined level of mortality (20%) calculated using a probit analysis. Of the two species, *C. vomitoria* larvae were the most tolerant to desiccation, experiencing 20% mortality after 2.7d (fig. 4.4); linear regression analysis of data from the trial assessing individual desiccation resistance (fig. 4.2) equates to *C. vomitoria* tolerating a 23.4% loss in water at 20% mortality. *Calliphora vicina* larvae were the least tolerant of water loss, and had experienced just 19.7% loss of water when 20% mortality was reached (9.2d, fig. 4.4). The cross was more desiccation tolerant than either of its parental species, tolerating a loss of 33.9% of water before 20% mortality was reached (4.3d, fig. 4.4).

Desiccation of groups of *C. vicina* for 7 days resulted in no discernible change in mortality over the 7 day period, with larvae maintaining 100% survival for the first 6 days of desiccation but experiencing 98% mortality when desiccated for 7 days (data not shown); as such a probit analysis to ascertain 20% mortality when *C. vicina* were desiccated in groups could not be generated. Desiccating *C. vomitoria* for 7 days at <5%RH did result in a reduction of survival rate (fig. 4.4) and a probit analysis revealed that 20% mortality was reached after 7.1d. As outlined above, analysis of data from group desiccation resistance trials (fig. 4.3b) indicates that larvae tolerate a 34.2% loss of water content before 20% mortality was reached.

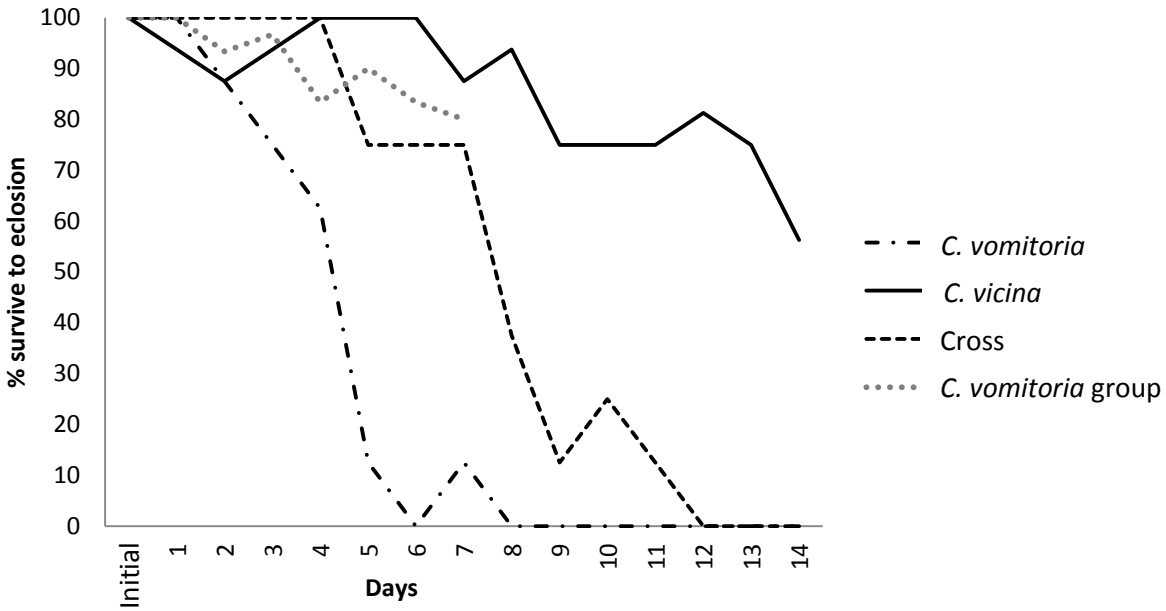


Figure 4.4. Mean survival to eclosion of individual L3 larvae of *C. vicina* (termed 'C. vicina'), *C. vomitoria* (termed 'C. vomitoria'), their cross (termed 'cross') and groups of *C. vomitoria* (termed 'C. vomitoria group') following different periods spent at <5% RH at 5°C. In the individual experiments, eight individual larvae of each of the three species were removed daily for 14 days. For group experiments, three vials each containing ten larvae of *C. vomitoria* were removed daily for 7 days. Once larvae were removed they were transferred to 20°C; *C. vicina* larvae were held in dry sawdust until eclosion occurred, *C. vomitoria* and the cross were placed in damp sawdust until eclosion occurred. Standard error is not included as only one trial was conducted using eight individual larvae.

4.4.2. Cross tolerance to cold

Calliphora vicina had a high eclosion success (87.5 ± 1.6%) in controls held at 5°C for 4d prior to a plunge at -9.5°C for 2h. Cold tolerance significantly increased (P=0.013) in desiccated samples (97.5 ± 1.6%) (data not shown). Mean survival to eclosion of *C. vomitoria* exposed to 5°C for 24h prior to a plunge at -13°C for 2h was 36.0% (± 7.5%) (fig. 4.5); the percentage of larvae surviving to pupation was higher (46.0 ± 6.7%). Cold tolerance was also significantly enhanced by prior desiccation in this species, increasing to 67.2% (± 4.69%) (P<0.001); the percentage of larvae surviving to pupation was higher (90.6 ± 3.1%).

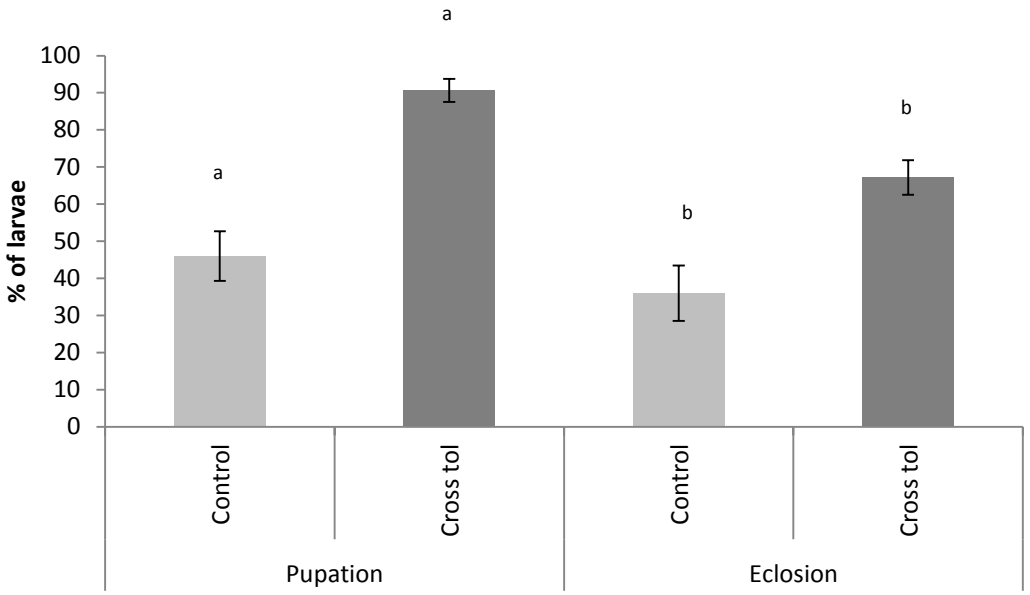


Figure 4.5. Mean percentage (±SE) of L3 *C. vomitoria* larvae pupating and eclosing after exposure to control and cross tolerance treatments. For control samples, glass vials containing eight larvae were held at 5°C for 24h and then exposed to -13°C for 2h (N=8), larvae were then held at 20°C until larvae eclosed. Cross tolerance (cross tol) treatment involved holding vials containing eight larvae in Drierite™ at 5°C for 24h prior to being exposing to -13°C for 2h (N=8). Groups with the same letter are significantly different to each other.

4.5. Discussion

Desiccation poses a real treat to survival of insects. Both *C. vicina* and *C. vomitoria* are common species in the UK and *C. vicina* is also known to be present from the sub-Arctic (Aak *et al*., 2011a) through to the sub-Antarctic (Lebouvier *et al*., 2011). During winter, especially at poleward latitudes, environmental ice can make water inaccessible and therefore desiccation may occur. The desiccation resistance, effect of group size on resistance, desiccation tolerance and cold tolerance were assessed in *C. vicina*, *C. vomitoria* and their cross.

The rates of water loss seen in both *C. vomitoria* and the cross were significantly higher than in *C. vicina*, suggesting that *C. vicina* is the most desiccation resistant; for example, after 10 days of desiccation at 5%RH, *C. vicina* had 80.5% of initial water remaining, whereas *C. vomitoria* and the cross had just 43.1% and 38.2%, respectively. The permeability of the arthropod cuticle is known to vary among species and between different life stages. Using gas chromatography, Roux *et al.* (2006) assessed the hydrocarbon profile of the cuticle in *C. vicina* and *C. vomitoria*. Although the profiles were distinct between the two species in most life stages, the

hydrocarbon profile of the larval cuticle was similar for the two species. Due to a similar hydrocarbon profile being detected, the large difference in desiccation tolerance seen between *C. vicina* and *C. vomitoria* in this current study suggests that the difference in water loss is determined by the spiracles rather than the cuticle. Larvae are able to selectively close their spiracles; previous studies have suggested that this closure is to relieve oxidative stress rather than to avoid desiccation (Hetz & Bradley, 2005). However, these findings have recently been disputed after research on the cockroach, *Nauphoeta cinerea*, which showed that when held at lower humidities, adults opened their spiracles for shorter durations and showed less body mass loss during respirometry than those held at higher humidities (Schimpf *et al*., 2009). The desiccation tolerance seen in this study is thought to be lower than that for other species held in similar conditions. For example, pupae of *S. crassipalpis* experienced a c. 10% water loss after 5d at 5%RH (Hayward *et al*., 2004); this was much lower than that seen in this study for either *C. vicina, C. vomitoria* or their cross, which lost 17.2%, 49.6% and 52.2% of their initial water content, respectively. This comparative increase in water loss in insects in the current study may be caused by the different life stages examined; *S. crassipalpis* were desiccated as pupae, which will affect the rate of cuticular water loss.

Within the laboratory, post-wandering larvae (having ceased active feeding and found a site to pupate) of *C. vomitoria* were seen to have an increased tendency to aggregate compared to *C. vicina* (personal observations). Aggregation is known to decrease respiration rate, for example in the firebug, *Pyrrhocoris apterus*, respiration rate was inversely related to the size of the aggregation (Yan-Le *et al*., 2007). In this study, groups of both *C. vicina* and *C. vomitoria* were seen to be more resistant to desiccation if held in groups rather than individually desiccated. *Calliphora vicina* remained the most desiccation resistant; larvae in groups did show a small (but significant) decrease in water loss, with 22.2% water loss when individual larvae were desiccated for 4d and 21.0% water loss when larvae were held in groups. *Calliphora vomitoria* were seen to have a 63.9% water loss when individual larvae were desiccated for 4d and just a

34.2% water loss when larvae were desiccated in groups. This alteration in water loss in *C. vomitoria* is in agreement with prior research by Benoit *et al*. (2007a), who identified that if *C. lectularius* were held in groups of 20 instead of individually, the rate of water loss per hour halved. Also, a 50% reduction in water loss was seen in *C. antarcticus* but this was recorded for a very large aggregation (600 adults; Benoit *et al*., 2008).

Gradual desiccation is known to affect the water loss and survival rate in organisms. In *D. octaedra,* when larvae are acutely desiccated for 14d, survival was low at c. 5%; however, when gradually dehydrated over the same period more than 90% of larvae survived (Petersen *et al.,* 2008). Although an alternative, higher RH was not established in this study; by grouping larvae together, a microclimate of a higher relative humidity may have induced a gradual rate of desiccation. Within the glass vials, larvae were arrayed in two layers; the RH on the top layer would have been close to the measured humidity of <5%, on the bottom layer a microclimate of a higher RH would have been created, which may have allowed larvae to dehydrate at a slower rate.

Although resistance to water loss is important in a species, the point at which water loss results in mortality is of critical importance. The greatest level of desiccation tolerance recorded in this study was seen in groups (aggregates) of *C. vomitoria*; larvae could undergo a 34.2% loss of initial water content before suffering 20% mortality. Of all individually desiccated larvae, surprisingly, the cross was found to be most tolerant of water loss, allowing 33.9% water loss before 20% mortality was reached; *C. vicina was* the least tolerant with just 19.7% water loss before suffering the same level of mortality. A class of organisms exist (anhydrobiotic) which are able to survive a large loss in water content with little effect on survival, for example the beetle, *Plytho deplanatus*, is able to withstand a 70% loss of initial water content during winter (Ring, 1982). Water content is known to decrease to 2% of initial body weight (Danks, 2000). When *C. vomitoria* were exposed to acute desiccation at <5% RH, a loss of just 40% water content led to

limited survival (<5%); the cross was able to tolerate a maximum water loss of 60% before 100% mortality was reached. The cave cricket, *Hadenoecus cumberlandicus*, was able to tolerate just a 26% loss of water content before complete mortality was reached, when exposed to <5%RH (Yoder *et al*., 2011). Desiccation tolerance to 50% mortality in *S. crassipalpis* was seen to be 24.5% of initial body mass when desiccated at 5%RH (Yoder & Denlinger, 1991); when data from this study is recalculated to be comparable, 50% of *C. vomitoria* failed to eclose after 25.2% loss of initial body mass and the cross after 41.9% loss of initial body mass. 50% survival was not reached in *C. vicina* so a comparison with this species cannot be made. Both *C. vomitoria* and the cross are more desiccation tolerant than either *H. comberlandicus* or *S. crassipalpis*, although they are not as desiccation tolerant as anhydrobiotic organisms.

The ability of organisms to display a cross-tolerance phenotype (desiccation and cold tolerance) is proposed to be due to the evolution of cold tolerance from desiccation tolerance mechanisms. In this study, a cross tolerance phenotype was recorded in both species, with larvae which had experienced desiccation prior to freezing showing higher survival than those which experienced freezing alone. The identification of the cross-tolerance phenotype is becoming increasingly widespread. For example, the nematode *Plectus murrayi* is able to increase its tolerance to 3d at -10^{o}C from c. 55% survival to 100%, if exposed to gradual dehydration (12h at 98%RH, 12h at 85%RH) prior to the freezing stress (post cooling from -4^{o}C to -10^{o}C at 1^{o}C.h^{-1}; Adhikari *et al*., 2010). Cross-tolerance to cold was seen in *B. antarctica,* which saw a c. 90% increase in survival if larvae were pre-treated for 48h at 98.2%RH before being exposed to sub-zero temperatures (48h at -10^{o}C) (Hayward *et al*., 2007). Cross-tolerance is not found in all species, for example in *Drosophila melanogaster* there was no correlation between survival of desiccation and cold tolerance (MacMillan *et al.,* 2009); after experiencing either 1h at 0 or-5^{o}C insects were placed inside a sealed container with 2g of silica gel and the number of hours until mortality was reached was recorded, it should be noted that the %RH was not recorded.

From this study it is clear that *C. vicina* are better able to resist water loss than *C. vomitoria*, but neither species exhibit characteristics of a truly anhydrobiotic organism, which are able to tolerate loss of up to 99% of normal water content (Crowe, *et al*., 1992). Both species were able to tolerate a modest amount of water loss before mortality reached 20%, therefore it is suggested that *C. vicina*, *C. vomitoria* and their cross are desiccation sensitive species. Furthermore, evidence has been presented in this study which supports earlier indications of an evolutionary link between cold tolerance and desiccation.

Calliphora vicina overwinters as third instar larvae in the soil. During winter larvae may experience extremes of temperature. Even within the U.K. water during winter can be inaccessible, locked away as snow or ice for weeks at a time. This could lead to increased mortality of overwintering larvae in the field, As insects as poikilotherms Temperature regulates the majority of development as such an increase or decrease in field temperature may have significant effects on the phenology of *C. vicina*. This has not, as of yet, been assessed in the field, during chapter 5 these questions are addressed.

5. Effect of climate change on diapause and the phenology of the blow fly, Calliphora vicina

5.1. Summary

The effect that increased temperatures due to global warming may have on diapause incidence, duration and termination was established in the blowfly, *Calliphora vicina* R-D., through field trials conducted in 2009 and 2010. Previous laboratory studies have suggested that the critical day-length (CDL) for *C. vicina* at 51°N is 14h 30min. The critical day length required to induce diapause in the field was seen to be correlated with civil dawn and dusk, as a low incidence of diapause was noted during August (0% in 2009, 2.3% in 2010) which is before CDL occurs. Eggs oviposited in October (post-CDL) showed a high incidence of diapause (up to 79%).

A cloche was used to simulate increased temperatures due to global warming and was compared to two current overwintering sites of *C. vicina*, an open field site and a woodland site. The cloche showed a 3.8 ±0.03°C increase in autumn/winter temperature 2009 and 2.9±0.03°C in autumn/winter 2010, compared to the open control site. This increase in temperature is thought to be within the range of the predicted rise in surface temperature over the next 150 years. The cloche site was seen to have a significantly lower percentage of larvae entering diapause (e.g. 15% in September 2009) when compared to both the open field (e.g. 63% September 2009) (P=0.007, over all trials) and the woodland (e.g. 58% September 2009) (P=0.004, over all trials). Diapause, in both the woodland and open field sites, was shown to be maintained by the majority of larvae until December (59.3 ±2.7%), with some remaining until March (0.84 ±0.5%). Over the 2009/2010 field trials, the majority of larvae in the global warming scenario did not enter diapause (86.8 ±3.7%), but for those that did, diapause was also maintained through to December (12.2 ±6.1%), with some remaining in February (4.0 ±3.65%).

Laboratory studies showed that diapause larvae required 24h at an increased temperature (25°C) to terminate diapause; lower temperatures (20°C and 15°C) required a longer period of time to result in diapause termination. Diapause larvae were also seen to be more cold tolerant than non-diapause larvae, with diapause larvae having an $LTemp_{50}$ at -7°C of 12.9d, whereas non-diapause larvae had an $LTemp_{50}$ of just 1.45d. Cold tolerance was further increased by acclimating larvae at 0°C for 7d prior to sub-zero temperature exposure, with diapause larvae increasing their $LTemp_{50}$ at -7°C to 14.6d and ND to 7.64d. Cold tolerance was also increased in ND larvae when the maternal generation was cultured at lower temperatures, suggesting that the maternal generation is capable of influencing the cold tolerance of their progeny. If the projected increase in temperature occurs due to climate change it is likely to result in a decrease in diapause incidence in *C. vicina*; as the cold tolerance of *C. vicina* has been shown to be lower in non-diapause larvae, this could lead to an increase in mortality if larvae are exposed to sub-zero winter temperatures.

5.2. Introduction

With the ongoing emissions of greenhouse gasses, global average surface temperature seems likely to continue rising. Clear effects of climate warming have been documented on plants, e.g. the Namib Desert tree, *Aloe*, (Zhu *et al.*, 2012), vertebrates, e.g. the polar bear, *Ursus maritimus* (Durner *et al*., 2011), and invertebrates, e.g. the small white butterfly, *Pieris rapae* (Gordo and Sanz, 2005). Indeed for insects, the effect of global warming is particularly important because they are poikilothermic, so rates of development and survival will be directly affected by temperature changes, which will in turn influence patterns of distribution and abundance both in space and time. The effects of climate warming upon insect distribution (Parmesan *et al.*, 1999; Takeda *et al.,* 2010), phenology (Roy and Sparks, 2000) and the synchrony, or loss of, between insects and food sources (van Asch *et al*., 2007) is being increasingly understood, but these studies have largely focused on impacts during the 'active season', i.e. spring through

summer. The effect that climate change has on overwintering invertebrates is less well understood, especially with respect to phenology and survival. Global average surface temperatures have risen a total of 0.8°C (±0.2°C) since 1850 (IPCC, 2007). However, average global temperatures are a poor guide to local or seasonal variability. A warmer than average global temperature may or may not mean a warmer than average temperature in the U.K.. Winter cold is still certainly a survival threat for U.K. insects, as was evidenced by the recent coldest winter on record, in 2009/2010. For insects to be successful, they must undergo a seasonal life cycle transition that matches development and reproduction to favourable environmental conditions, and diapause/dormancy with periods of food shortage and/or low temperature stress (see fig. 5.1). This life cycle transition pivots around the programming of diapause by photoperiod (Vinogradova & Zinovjev, 1972), but can be disrupted by temperature (fig. 5.1; Bale & Hayward, 2010). For example, holding the blow fly, *Calliphora vicina* (R-D.) at temperatures above 12 to 17°C, dependent upon geographical location, causes larvae to avert diapause and pupariate (Vinogradova & Zinovjev, 1972; Saunders, 1987; Chernysh et al, 1995; McWatters & Saunders, 1998). Consequently, the impact of climate change will be determined by whether temperature increases decouple diapause from seasonal changes.

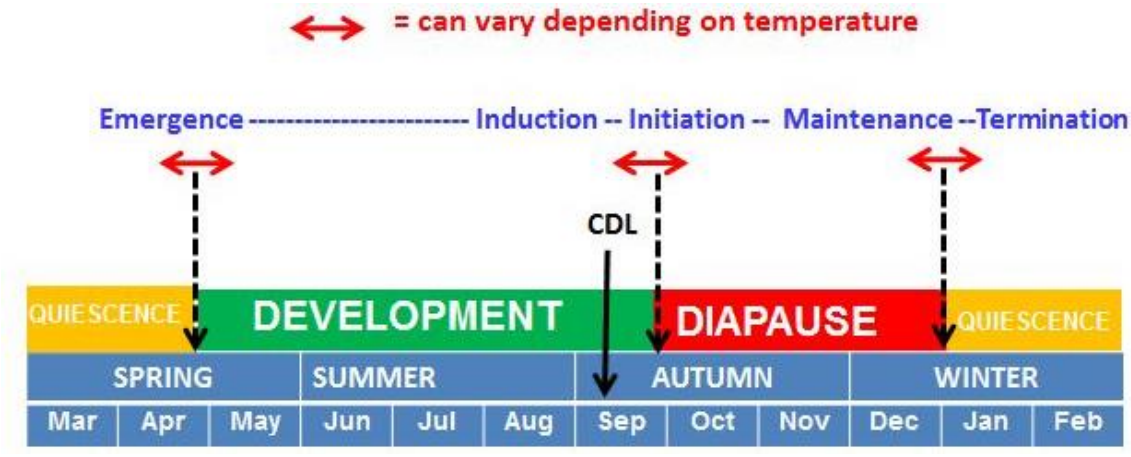


Figure 5.1. Schematic depiction of temporal development of *Calliphora vicina*, including development (green block), diapause (red block) and post-diapause quiescence (yellow block; termed quiescence). Dark sold arrow represents the current critical day length (CDL), which occurs in early Autumn. The dotted arrows represent the timing of the transition between the different stages, each of which is thought to vary depending on temperature (red arrows). Figure modified from Hayward *et al*., unpublished.

Diapause is a pre-emptive dormancy which is genetically programmed to occur at a specific stage of an insect's life cycle and the stage differs from species to species. Most insects have a facultative diapause which is induced by specific environmental cues, such as photoperiod, experienced during a 'sensitive stage' of development (see chapter 1). For example, in the blow fly, *C. vicina*, the sensitive stage occurs in the adult generation, and if the critical day length (CDL – 14.5h at 51^{o}N) is experienced at this stage, their progeny will enter diapause as third instar (L3) larvae (McWatters and Saunders, 1996). This maternal regulation of diapause is common among insects (Tauber *et al*., 1986), including the Hymenopterid *Trichogramma ambryophagum* (Raznik *et al*., 2011), the rice leaf bug, *Trigonotylus caelestialium*, (Shintani, 2009) and the blow fly, *Lucilia sericata* (Tachibana & Numata, 2004), and represents a transfer of environmental information across generations. Determination of the CDL is undertaken in the laboratory, and this value is then used to estimate the seasonal timing of diapause induction under field conditions. However, there is no reference to when this would occur in the field. There are 4 definitions for length of photoperiod: sunrise to sunset, which both occur when the centre of the sun is 0.83^{o} below the horizon (accounting for apparent solar diameter and atmospheric diffraction) and also 3 definitions that include a twilight interval. Twilight definitions are: astronomical twilight, which is defined as the period that the centre of the sun is between 18^{o} and 12^{o} below the horizon; nautical twilight in which the solar centre is between 12^{o} and 6^{o} below and civil twilight (commonly known as dawn/dusk) in which the solar centre is between 6^{o} and 0.83^{o} below the horizon. Laboratory studies on the Dipteran fungus gnat, *Bradysia* sp. nr. *coprophila,* was seen to positively respond to light stimulus when light intensity rose above 6.95 lux (Cloyd *et al*., 2007); on a clear day, the beginning of civil twilight produces c. 3 lux and sunrise produces c. 400 lux (Johnsen *et al.,* 2009), though the lux at which photoperiod is stimulated is currently unknown. Consequently, we have limited direct evidence of when diapause is actually induced under field conditions.

Temperature also plays a critical role in regulating diapause because it determines the rate of insect development. Thus, a potential impact of climate warming is that the sensitive stage becomes out of synchrony with the seasonal timing of the CDL. For example, the commonly univoltine leaf beetle, *Phratora vulgatissima,* has a facultative diapause induced by decreasing photoperiod, and has a CDL of 18h 10min in Uppsala, central Sweden (59^{o}N) (Dalin, 2011). In recent years, first-generation beetles have been observed to mature before the CDL is reached, due to higher spring and summer temperatures, allowing a second generation to develop which subsequently entered a reproductive diapause (Dalin, 2011); however, comparisons between the winter mortality of the uni- and bi-voltine groups are yet to be assessed. The pitcher plant mosquito, *Wyeomyia smithii*, has also been affected by increasing temperatures, particularly along its northern boundary, where the mosquito has lengthened its CDL over a 5-30 year period to remain in synchrony with the onset of winter conditions (Bradshaw and Holzapfel, 2001). In addition, elevated temperatures can result in diapause being averted, even under appropriate photoperiodic conditions. For example, in the adult diapause of the exotic beetle, *Zygogramma bicolorata,* diapause can be averted if exposed to 35^{o}C when newly emerged (Sushikumar & Ray, 2010).

Diapause duration (also termed intensity, strength or depth) is known to vary at both the species and individual level (Beck, 1980), with diapause lasting from a few weeks in *L. sericata* (Tachibana & Numata, 2004) to up to 30 years in the Yucca moth, *Prodoxus Y-inversus,* (Powell, 2001). The duration of diapause is initially defined during the induction stage, with photoperiod and temperature being the primary drivers (McWatters and Saunders, 1998); however, many species remain receptive to these cues whilst in diapause. For example, when diapausing larvae of *Sesamia nonagriodies* were transferred to a long photoperiod, all larvae pupariated, regardless of the photoperiod and temperature of diapause induction (Fantinou *et al*., 2003). Temperature can also have a dramatic influence on diapause duration, as was seen in the Mediterranean corn borer, *Sesamia nonagrioides,* (Eizaguirre *et al.,* 2008), the flesh fly, *S.*

bullata, (Denlinger, 1972) and the cotton bollworm, *Helicoverpa armigera,* which saw a 69% increase in diapause duration when held at 21°C rather than 23°C (Wu *et al*., 2010), this alteration of diapause duration will have effects on the start of post-diapause quiescence (fig. 5.1).

Currently, in temperate regions, insects typically terminate diapause in mid-winter, while adverse conditions are still continuing (Hodek, 1996); for example, the Coleopterid *Aulacophora nigripennis* terminates diapause in January or February (Watanabe & Tanaka, 1998) and the psyllid *Strophingia ericae* terminates diapause in December or January (Miles *et al*., 1998). After diapause is terminated, insects can enter post-diapause quiescence (fig. 5.1), during which stress tolerance mechanisms can be retained, e.g. in the flesh fly, *S. crassipalpis* (Hayward *et al*., 2005). Termination of quiescence and the resumption of development occur when environmental temperature exceeds the developmental threshold for the insect. The developmental threshold for the predatory mite *Amblyseius californicus* and its prey *Tetranychus urticae* is at 9.9°C and 8.6°C respectively (Hart *et al.,* 2002), 9.8°C in the blow fly, *Protophormia terraenovae* (Grassberger & Reiter, 2002); and somewhere between 1°C (Donovan *et al.,* 2006) and 6°C (Haskell *et al*., 2000) in *C. vicina*. If temperature increases beyond their developmental threshold, e.g. false springs, insect emergence can occur and may result in dramatic negative consequences if not in synchrony with environmental factors such as food, or with other insects of the same species to allow mating. Whilst there is considerable evidence that high temperatures can result in early termination, we have a limited understanding of what durations of exposure to high temperatures will trigger this response, yet this information is critical in order to use climate data to model where and when diapause may be disrupted.

If diapause is averted as a result of autumn conditions, i.e. at the time of experiencing the CDL, then non-diapause stages will likely be exposed to winter cold because another life cycle cannot be completed in order to return to the diapause stage before winter (Bale & Hayward, 2010). This will impact on overwintering survival, and can determine the distribution limits of some

species; for example, the northern boundary of the southern green stinkbug, *Nezara viridula*, is determined by the minimum January temperature (Musolin, 2007), and the predaceous flower bug, *Orius strigicollis,* population density is highest in urban areas of eastern Japan where winter temperatures are less severe due to urban 'heat islands' (Shimizu, *et al.,* 2001). As outlined earlier, it is already known that adult temperature can influence the incidence of diapause in *C. vicina* (Vinogradova & Zinovjev, 1972; McWatters & Saunders, 1998), but it is not known whether the adult's thermal history influenced the cold tolerance of their progeny. Linked to this, we do not know whether the epigenetic transfer of thermal gradients in *C. vicina* is only transferred as part of the diapause programme, or whether it can occur in non-diapause progeny. To determine this, it is necessary to compare the stress tolerance of diapause and non-diapause individuals from adult generations with different thermal histories. This will allow us to assess likely winter survival under different climate scenarios and where diapause may or may not be averted. The difference in cold tolerance between diapause and non-diapause larvae has been previously described; an increased tolerance to -8^{o}C in *C. vicina* (55^{o}N) diapause larvae was described by Saunders & Hayward (1998) who found that 50% survival of non-diapause larvae occurred at c. 2.5d, whereas diapause larvae could survive c. 7 days. In the two spotted spider mite, *Tetranychus urticae*, diapause larvae had an increased cold tolerance, having an $LTemp_{50}$ of -19.7^{o}C compared to non-diapause larvae at just -13.3^{o}C (Khodayari *et al*., 2012). An increase in tolerance to sub-zero temperatures was also seen in diapause larvae of the pine caterpillar, *Dendrolimus tabulaeformis*, (Han *et al*., 2005).

Specifically, the research in the chapter addresses the following questions: When is diapause induced in the field? How does temperature affect diapause initiation in the field under current thermal conditions and under climate warming scenarios? Will diapause duration and termination be affected under simulated global warming scenarios? What is the cold tolerance of both diapause and non-diapause larvae, and does adult thermal history affect the cold tolerance of larval progeny?

5.3. Methods

5.3.1. Field cultures

A sample of approximately 70 adult flies was placed inside a metal insect cage measuring 30cm per side within the University of Birmingham grounds (grid reference SP049843). Water and sugar were provided in excess at all times. Liver was provided for 24h on days 4, 6, and 8 after eclosion, and every day thereafter. Eggs were collected on the first day of mass oviposition after day 12, as Saunders (1987) identified that diapause incidence is greatest after 12 photoperiodic cycles (see chapter 2 for further details). The egg mass was observed and the number of days to mass oviposition was recorded. The liver and eggs were placed in a damp airtight container for 24h, next to the cage of adults in the field, so that a high number of first instar (L1) larvae could be produced. A Tinytalk® datalogger (Gemini, UK) was placed within the cage and recorded the temperature every 3min throughout the field trials. Larvae obtained from these field cultures were used as outlined in the following sections. The cages were observed every second day to monitor for possible adult diapause, as was suggested by Vinogradova (1986).

5.3.2. Seasonal timing of diapause induction under field conditions

To determine the seasonal timing of diapause induction in *C. vicina* under field conditions, the incidence of larvae diapause was assessed in cultures from cages of adults set up on the following dates: 28th August, 14th September and 9th October. The first trial was initiated after the CDL (14.5h) was reached, if it were to be defined by sunrise and sunset (CDL defined by sunrise to sunset is reached on 19th August). The second trial was initiated on 14th September, which is after a CDL extending from civil dawn and dusk is reached (CDL of civil dawn till dusk is reached on 5th September). The final trial was initiated on 9th October, which is after the CDL extending from nautical dawn to nautical dusk (CDL of nautical dawn till dusk is reached on 25th September). These three trials were conducted in 2009 and then repeated in 2010 on the same

dates. As a control for the possible effect of temperature in the field being high enough to avert diapause, control groups of larvae (three replicates of n=10) were returned to the lab at constant 11^{o}C conditions, which should ensure a high incidence of diapause if the CDL had been reached.

Adults were fed as described in section 5.3.1 and the resultant L1 larvae (three replicates of n=20) were placed on 15g of liver wrapped in foil, which was placed on top of 5cm of sieved compost, all enclosed in a ventilated plastic container (fig. 5.2). A depth of 5cm of compost was used to mimic that which Callliphorids bury to under semi-natural conditions (Gunn & Bird, 2011). The plastic containers were buried at soil level and a data logger, recording temperature at three minute intervals, was placed at a depth of 5cm next to the containers. By placing the data logger at 5cm an accurate temperature of where the larvae were in the soil column was gained, it also buffered from any extremes of air temperature experienced above ground level. Larvae were located in an open field site (fig. 5.3a) and observed at day 30 (post oviposition) to assess the percentage of larvae that were in diapause (those that had not pupariated by day 30 post oviposition - Saunders, 1987).

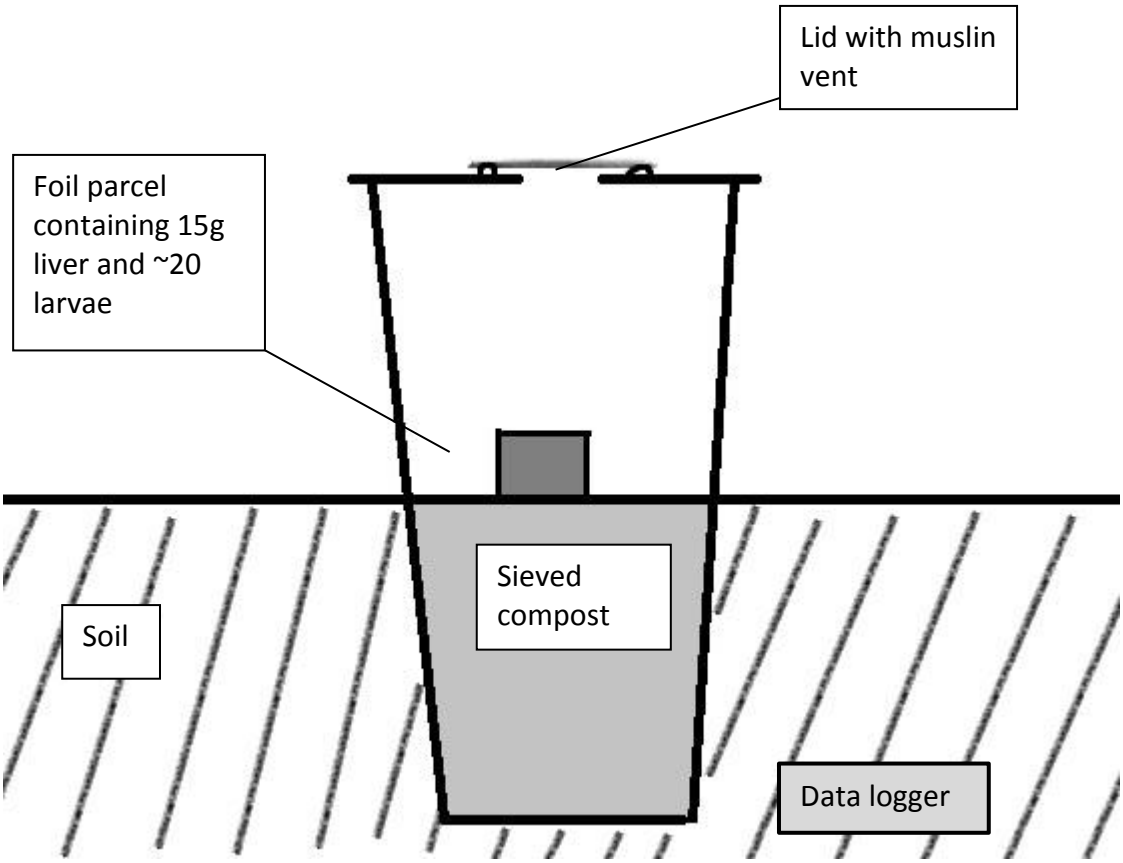


Figure 5.2. Schematic of the construction of containers used to house larvae used in all three field sites (open, woodland and cloche).

5.3.3. The effect of wintering site microclimate on diapause incidence

Three separate field sites were established to determine the effect of microclimate temperature on diapause incidence in *C. vicina*:

- 'Open' field site: area on the edge of a small ploughed field, roughly 50m away from a hedge (termed 'open site', fig. 5.3a).
- Woodland: situated 15m within the boundary of a small mixed deciduous wood ('woodland site', fig. 5.3b)
- Cloche: a glass topped cold frame, on a 30cm – deep bed of gravel ('cloche site', fig. 5.3c)

The first two represent overwintering sites already used by Calliphorids, and would allow assessment of how overwintering site selection can influence phenology. The third site provided a simulation of climate warming.

As described in section 5.3.1, larvae collected from adult field cultures (at all three dates, 28th August, 14th September and 9th October in both 2009 and 2010) were reared at all three of the sites (open, woodland and cloche). Data loggers, recording temperature at 3 minute intervals, were placed in the soil at each site, at a depth of 5cm.

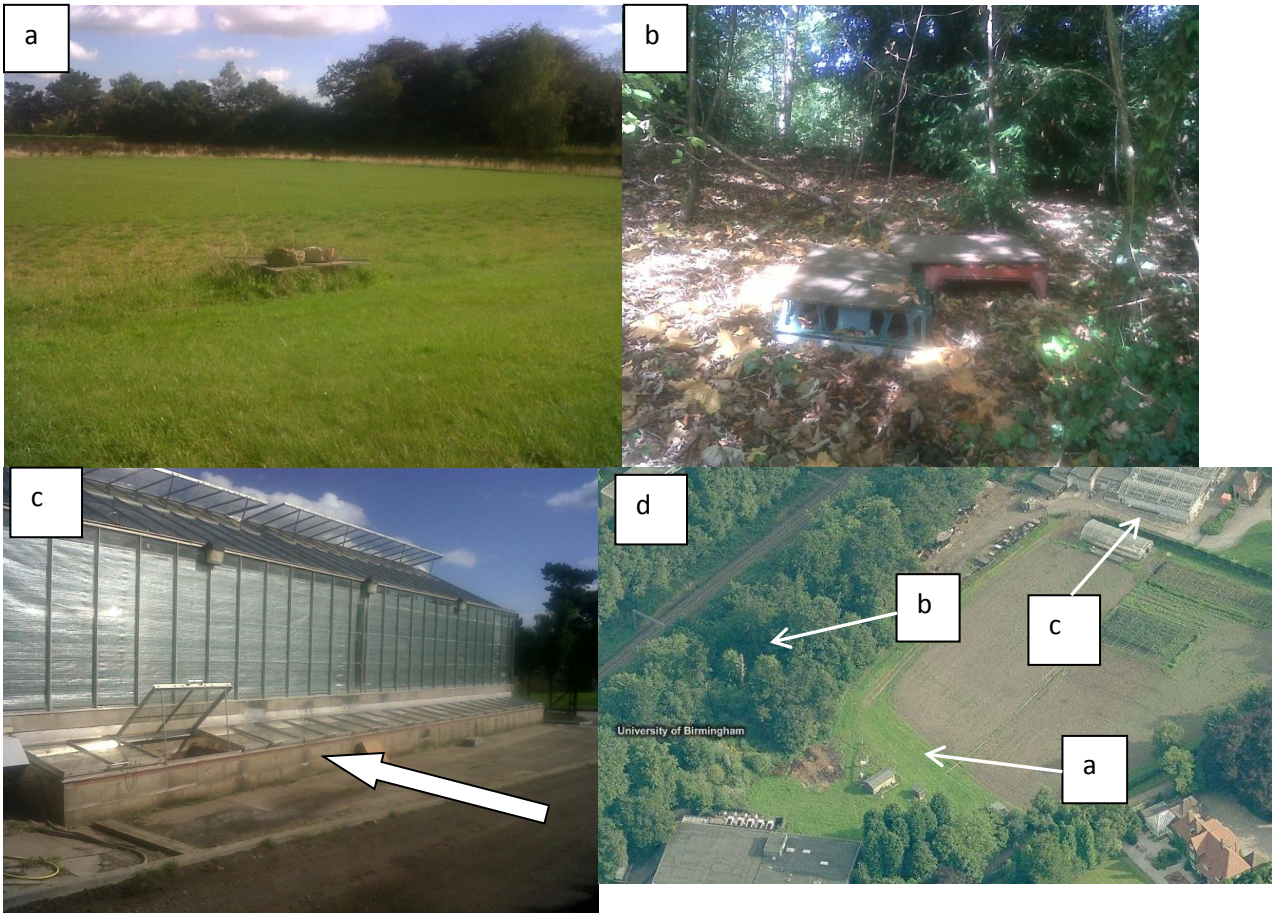


Figure 5.3. a) open site; b) woodland site; c) cloche site, d) aerial photo shows the location of the three sites in relation to each other. All located on the University of Birmingham campus, U.K.

5.3.4. The effect of temperature on diapause duration

5.3.4.1. The influence of winter microclimate

At each of the sites, larvae cultures were assessed each month to determine diapause duration. This was achieved by recording the number of *C. vicina* that were remaining as larvae (i.e. those that had not pupated).

5.3.4.2. Duration of diapause under different constant temperatures

As a comparison with the 3 different thermal regimes experienced at each field site, larval cultures programmed for diapause in the laboratory (see chapter 2 for more details) were set up in incubators at the following constant temperatures: 5, 11, 15, 20 or 25^{o}C. Diapause larvae were held at 11^{o}C in constant darkness from oviposition until 30 days post oviopsition, which was designated as day 0 of diapause (D0) (Saunders, 1987). Five petri dishes containing 10 D0 larvae in sawdust were transferred to each temperature and maintained under constant darkness (DD). Larvae were observed daily and the number of days to 50% pupariation recorded.

5.3.4.3. Duration of exposure at elevated temperatures required to terminate diapause

To determine what duration of exposure to elevate temperatures is sufficient to terminate diapause in *C. vicina,* D0 larvae were exposed to 15, 20 or 25^{o}C for 20mins, 1h, 2h, 6h, 1d, 2d, 4d or 7d. Following these exposures, all larvae were returned to 11^{o}C (DD) and the number of days taken to reach 50% pupariation was recorded by daily observation. To determine whether the sudden change in temperature had an effect on survival, the percentage of morphologically normal adults eclosing was recorded.

Whether the sensitivity of diapausing larvae to high temperatures changed during the course of diapause was assessed by repeating the above experiment using D10 diapause larvae (=d40 post

oviposition). For all experiments, larvae were kept in petri dishes with a small amount of sawdust. Sample sizes were n=60 for all experiments, with the exception of D10 larvae held at 15°C in which a sample size of n=30 was used.

5.3.5. Difference in the cold tolerance of non-diapause (ND) and diapause (D) larvae, and the influence of the parental generations thermal history

Lower Lethal Times (LT_{50}) were assessed for ND and D larvae at -7°C. L3 larvae were equally divided into 18 glass vials (2x3.5cm, N=10 per vial), which were then placed in trays of antifreeze, maintained at -7 $\pm$1°C (Fryka B30-20, Kältetechnik Esslingen). After 24h (and every 3d afterwards), 3 vials were removed and placed at 11°C for 3d, before being transferred to 20°C until eclosion. Initial survival was assessed 24h after removal from the antifreeze by determining whether larvae responded to a mechanical stimulus. Larvae were then observed every second day and the percentage eclosion success (completely extricated from pupal casing) recorded.

To assess the effect of acclimation on the cold survival of *C. vicina* ND and D larvae, groups of post-wandering L3 larvae were maintained for one week at 5°C (acc5) or 0°C (acc0). ND larvae were assessed 14 days post oviposition and D larvae were assessed 30 days post oviposition (D0 of diapause). LT_{50} values were assessed, as described above, and compared with samples transferred directly to -7°C. The influence of the thermal history of the parental generation (adults) on LT_{50} values of ND and D larvae was determined by rearing adult cultures at either 15°C or 20°C under either long day (L:D 18:6) or short day (L:D 12:12) conditions, to produce ND or D larvae respectively. The cold tolerance of their progeny was assessed as described above. Finally, to determine whether the cold tolerance of D larvae changed during the course of diapause, and whether this was influenced by adult thermal history LT_{50} values were also assessed for D10 and D20 diapause larvae from both 15 and 20°C cultures.

5.3.6. Statistical analysis

To determine whether the simulated global warming conditions (cloche) affected the proportion of *C. vicina* entering diapause when compared to the two current climate hibernation sites (wood and open), a student's t-test was performed in SPSS (SPSS v. 18.0).

To examine whether holding larvae at different constant temperatures significantly affected the number of days to pupariation, a Mann Whitney U test was performed in SPSS, as data was found to have unequal variance (Levenes test, $P<0.0001$).

To assess whether time or temperature had an effect of the proportion of larvae terminating diapause, a Kruskal-Wallis test was performed, as data between groups was not found to be homogeneous (K-S test, $P<0.001$). A pairwise Mann-Whitney U test was used for comparisons within groups.

The significance of any intergroup differences between cold tolerance (LT_{50}) data was analysed by non-overlapping 95% fiducial limits (Hart *et al.,* 2002; Hughes *et al*., 2009), as a result of probit analysis performed in SPSS.

5.4. Results

5.4.1. The seasonal timing of diapause induction under field conditions

Adult cages established in August (2009 and 2010) produced larvae with a very low incidence of diapause, 0% in 2009 and 2.3% in 2010. For cages set up in September, diapause incidence was 63% in 2009 and 79.1% in 2010 (fig. 5.4.). Mean daily temperatures were only above 15^{o}C in August 2009; however, there were periods where maximum air temperatures exceeded this value for brief periods in all months (Table 5.1.). Linked to this, the number of days to oviposition was shortest in September and greatest in October (fig. 5.5). The soil temperatures at 5cm were recorded for 30 days post oviposition for each of the field trials (Table 5.2).

Although the mean soil temperature during all trials never exceeded 15°C, the maximum soil temperature did for three trials, those started on the 28th August during both 2009 and 2010 and the 14th September trial in 2009. Control samples in which larvae oviposited from August field trials were held in the laboratory at 11°C; larvae showed a low incidence of diapause 6.6 ± 1.4% (N=60).

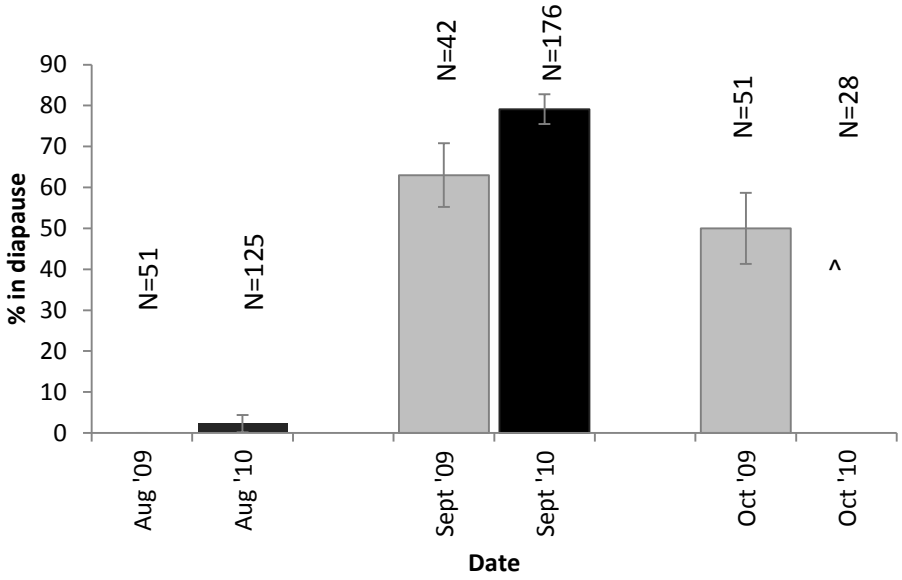


Figure 5.4. The mean percentage (±SE) of larvae in diapause at 30 days post-oviposition. Cages were established on 28th August, 14th September and 9th October in 2009 (grey bars) and 2010 (black bars). ^ designates trials where the percentage of larvae in diapause could not be recorded due to larvae being at the second instar stage, not the third instar in which diapause occurs.

Table 5.1. Minimum, mean and maximum air temperatures (°C) experienced inside adult cages during field trials from August 2009 to October 2010.

	Aug '09	Aug '10	Sept '09	Sept '10	Oct '09	Oct '10
Minimum (°C)	9.2	6.0	8.9	4.4	5.0	3.7
Mean (°C)	15.5	14.9	14.9	13.5	11.7	9.2
Maximum (°C)	36.6	35.7	26.2	35.0	26.9	22.0

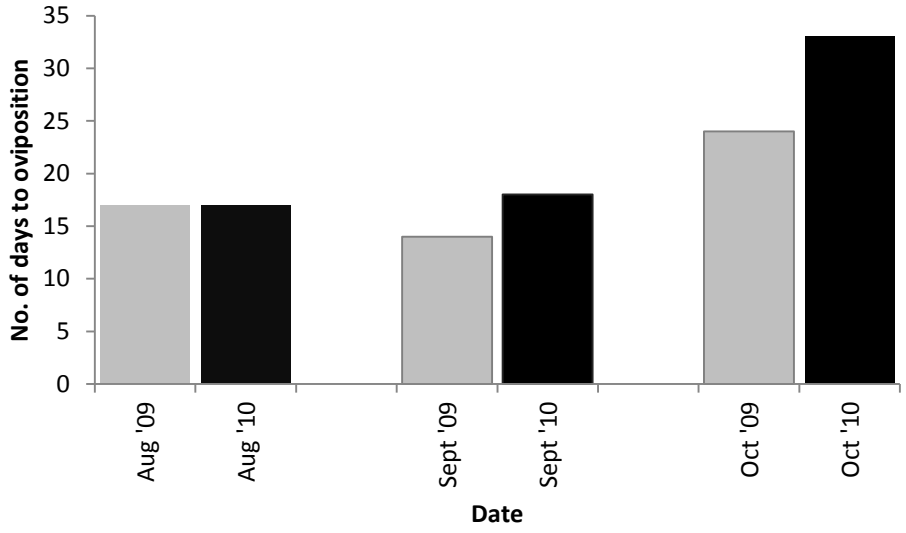


Figure 5.5. Number of days for adults to oviposit in field cages set up on 28th August (termed 'Aug'), 14th September (termed 'Sept') and 9th October (termed 'Oct') trial in 2009 (grey) and 2010 (black) (N=1 cage per trial).

Table 5.2. Minimum, mean and maximum soil temperature (°C) at 5cm at the open site in field trials conducted between August 2009 and October 2010. Soil temperatures were recorded from the date of oviposition for the next 30 days, for each trial.

	Aug '09	Aug '10	Sept '09	Sept '10	Oct '09	Oct '10
Minimum (°C)	8.1	9.6	8.1	5.0	2.5	-0.9
Mean (°C)	13.4	12.8	11.3	10.4	8.1	3.4
Maximum (°C)	26.2	18.4	19.3	14.2	11.0	9.0

5.4.2. The effect of wintering site microclimate on diapause incidence and duration

The results from the larval progeny of October 2010 cages are different from other dates because very low temperatures delayed development to the L3 stage. Larvae had high mortality before diapause and did not reach the L3 stage until January 2011. For this reason the data from October 2010 were excluded from further analysis.

Larval diapause was recorded in all trials, with the exception of August 2009 in which no larvae entered diapause at any of the sites (data not shown), the maximum, minimum and mean soil

temperatures during the larval development of the August field trial are shown in fig. 5.6. Diapause incidence was consistently highest in field trials established in September, compared to those created in either August or October in either 2009 or 2010 (fig. 5.7b. to fig. 5.10b).

Diapause incidence at the cloche site was consistently below 40% and significantly lower than for field and woodland sites (Student's T-test, P=0.007 and P=0.004, respectively). It also had the highest mean temperature during every month that was recorded (fig. 5.6, fig. 5.7a to fig. 5.10a). For the two current alternative diapause sites of *C. vicina* (open and wood), in three of the five field trials where insects entered diapause (September 2009 – fig. 5.7b, October 2009 – fig. 5.8b and October 2010 – fig. 5.10b), the percentage of larvae entering diapause was highest at the site with the highest temperature.

Diapause only persisted until February in larvae at the field or woodland sites, and very few individuals remained in diapause until March (2.6% (n=2) in woodland site from October 2009 cages and 1.8% (n=2) from open site from September 2010 cages).

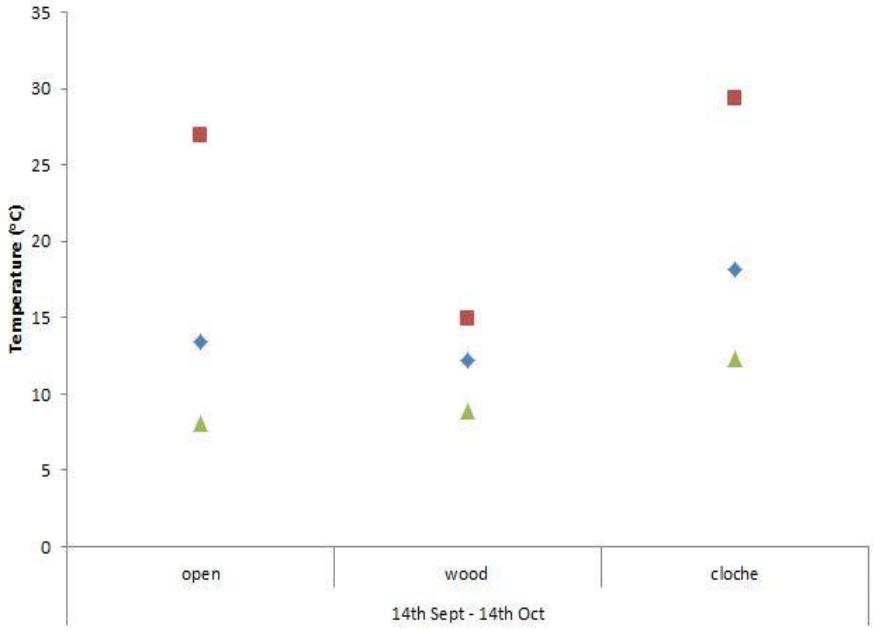


Figure 5.6: August 2009 trial. The maximum (red square), minimum (green triangle) and mean (blue diamond) soil temperatures at 5cm sampled every 3 minutes at the open site, wood site and cloche, assessed from 14th September 2009 to 14th October 2009.

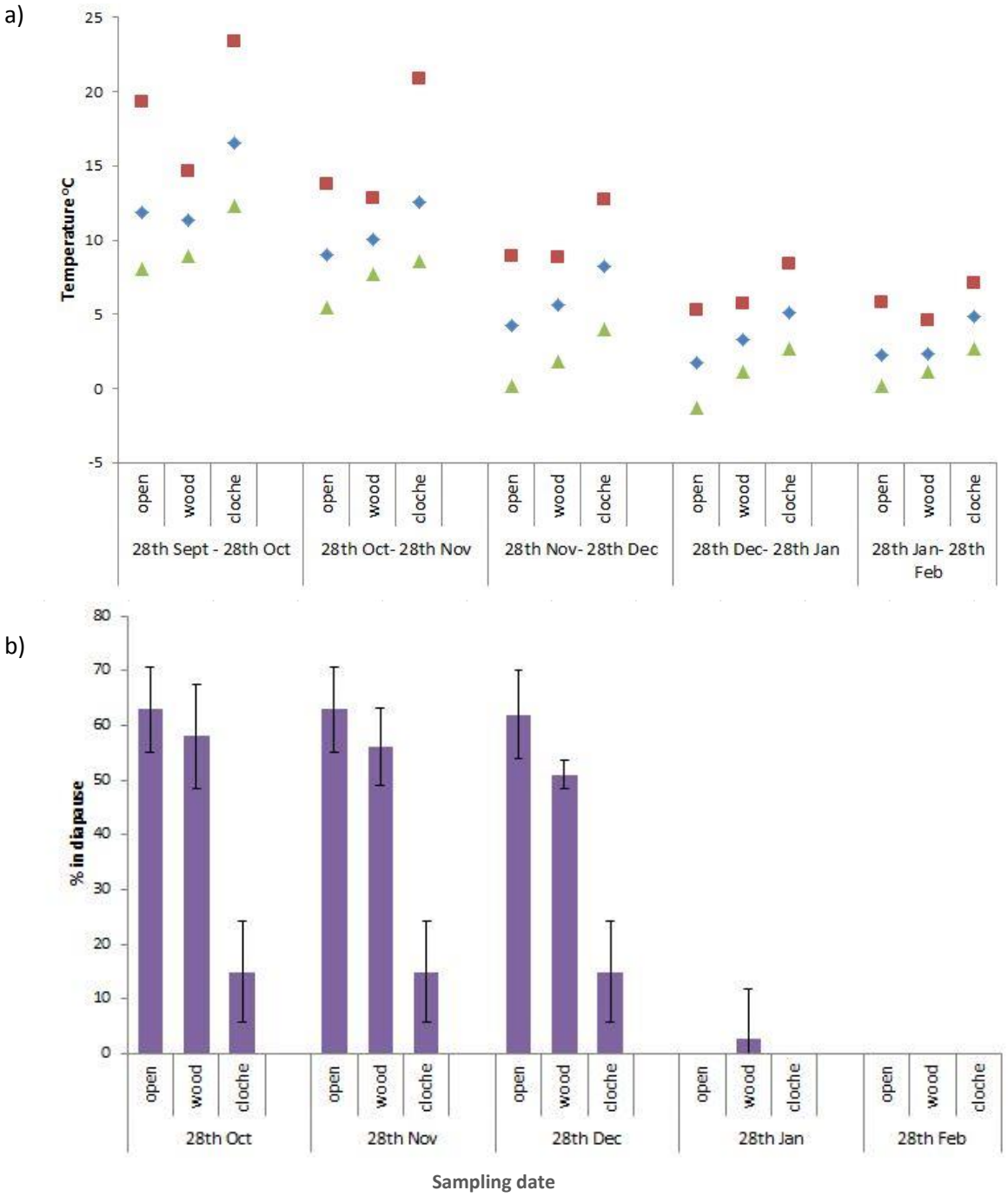


Figure 5.7: September 2009 trial a) The maximum (red square), minimum (green triangle) and mean (blue diamond) soil temperature at 5cm sampled every 3 minutes at the open site, wood site and cloche and summarised over monthly intervals from 28th September 2009 to 28th February 2010. b) The mean percentage (±SE) of *C. vicina* in diapause at the open (N=42), wood (N=45) and cloche sites (N=60) sampled between 28th October and 28th February. Adult cultures were established on 14th September 2009, eggs oviposited on 28th September and percentage diapause assessed on 28th October; and then every month thereafter.

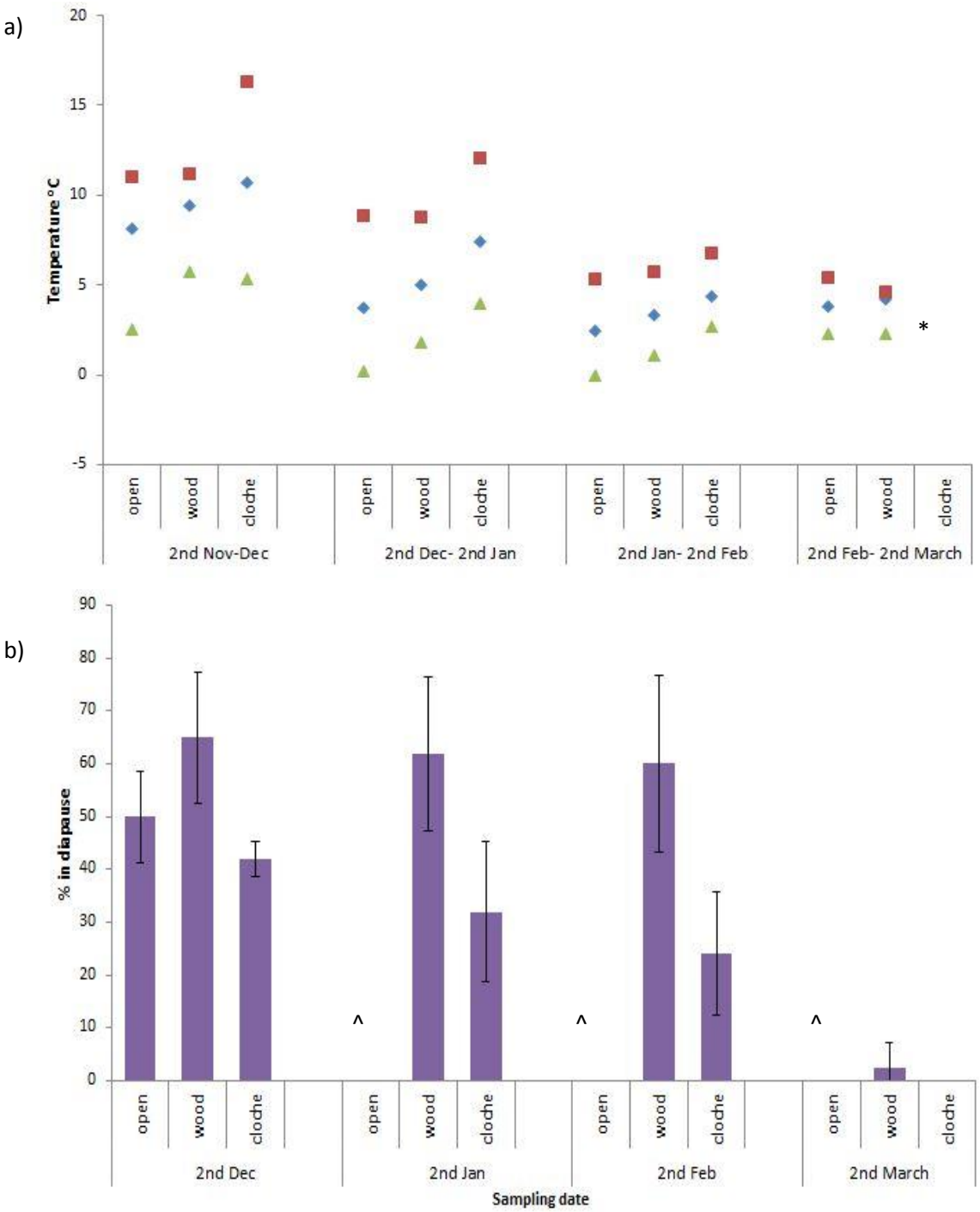


Figure 5.8: October 2009 trial a) The maximum (red square), minimum (green triangle) and mean (blue diamond) soil temperature at 5cm sampled every 3 minutes at the open site wood site and cloche and summarised over monthly intervals from 2nd November 2009 to 2nd March 2010. b) The mean percentage (±SE) of *C. vicina* in diapause at the open (N=51), wood (N=36) and cloche sites (N=60) sampled between 2nd December and 2nd March. Adult cultures were established on 9th October 2009, eggs oviposited on 2nd November and percentage diapause assessed on 2nd December; and then every month thereafter. ^ designates where samples could not be recorded due to loss of larvae through predation. * designates where data was not recorded.

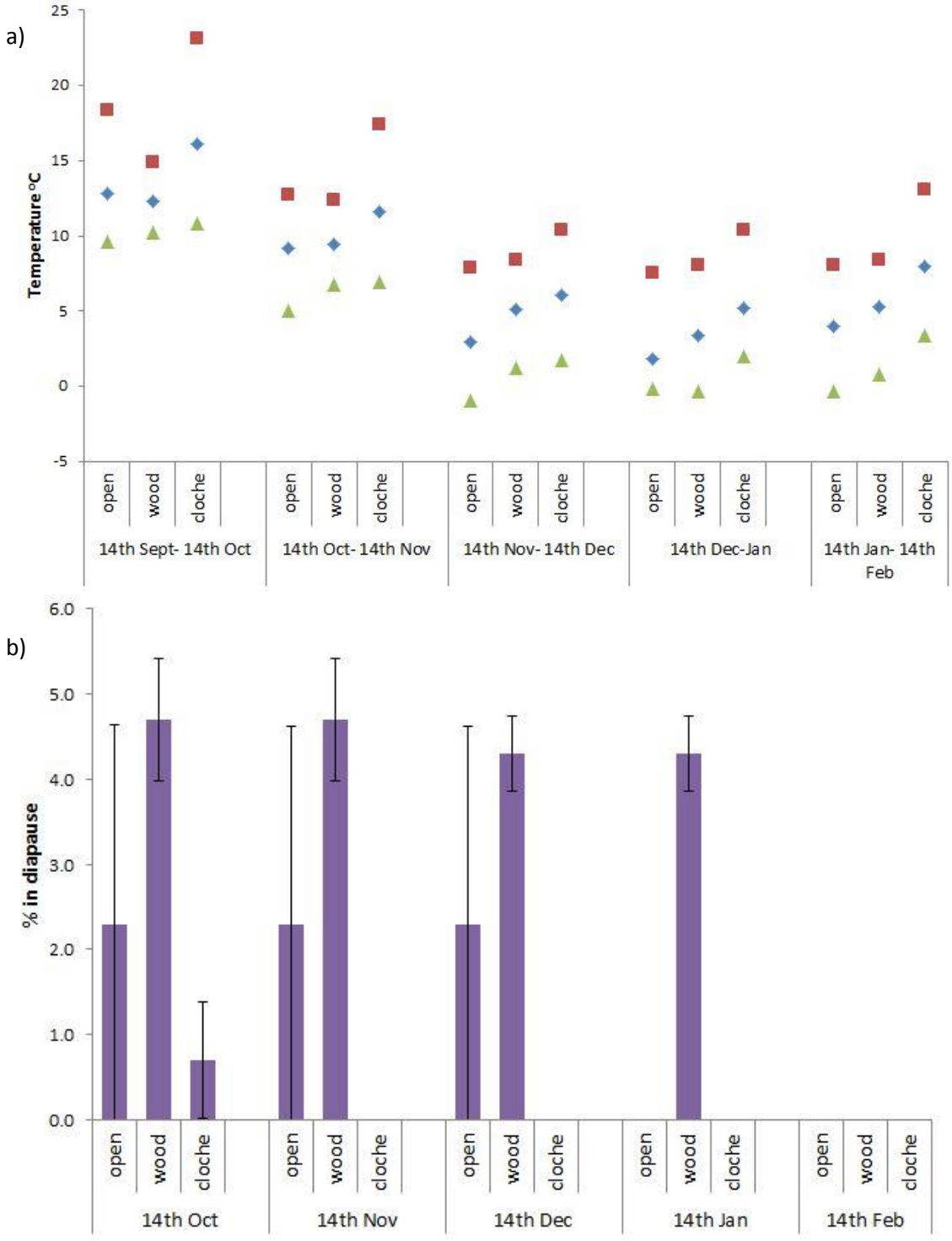


Figure 5.9: August 2010 trial a) The maximum (red square), minimum (green triangle) and mean (blue diamond) soil temperature at 5cm sampled every 3 minutes at the open site, wood site and cloche and summarised over monthly intervals from 14th September 2010 to 14th February 2011. b) The mean percentage (±SE) of *C. vicina* in diapause at the open (N=125), wood (N=192) and cloche sites (N=161) sampled between 14th October 2010 and 14th February 2011. Adult cultures were established on 28th August 2010, eggs oviposited on 14th September and percentage diapause assessed on 14th October; and then every month thereafter.

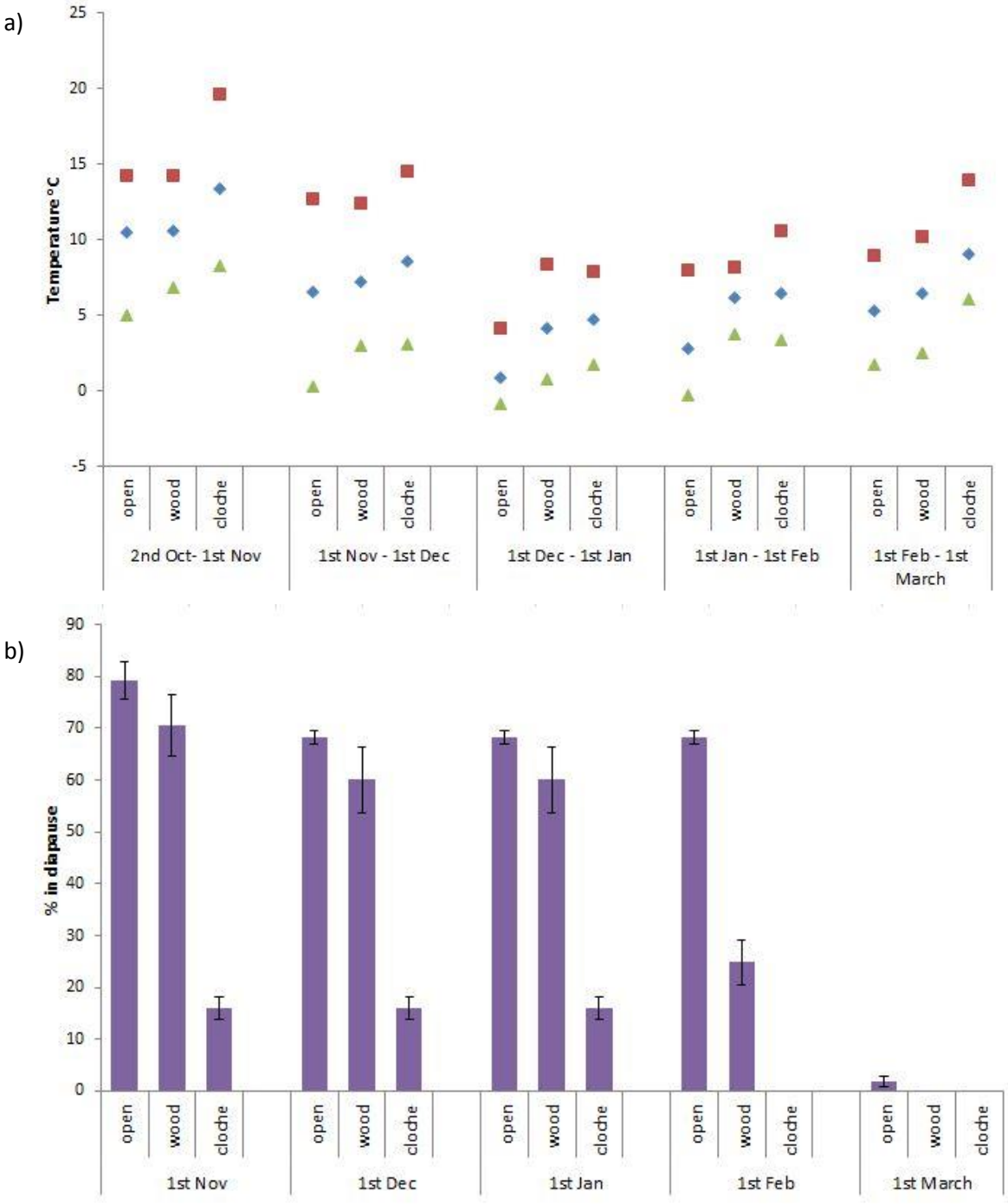


Figure 5.10: September 2010 trial a) The maximum (red square), minimum (green triangle) and mean (blue diamond) soil temperature at 5cm sampled every 3 minutes at the open site wood site and cloche and summarised over monthly intervals from the 2nd October 2010 to the 1st March 2011. b) The mean percentage (±SE) of *C. vicina* in diapause at the open (N=176), wood (N=156) and cloche (N=138) sites sampled between the 2nd November 2010 and the 1st March 2011. Adult cultures were established on the 14th September 2010, eggs oviposited on the 2nd October and percentage diapause assessed on 1st November; and then every month thereafter.

5.4.2.1. Diapause duration under different constant temperatures

Overall trends show a general decrease in the number of days spent in diapause with increasing temperature (fig. 5.11). The number of days to pupariation differed significantly between all

temperature groups (P>0.001, Student's T-test, N=50 for all groups), with the only exception being the number of days to pupariate between 20°C and 25°C (P=0.752, Student's T-test, N=50 for each).

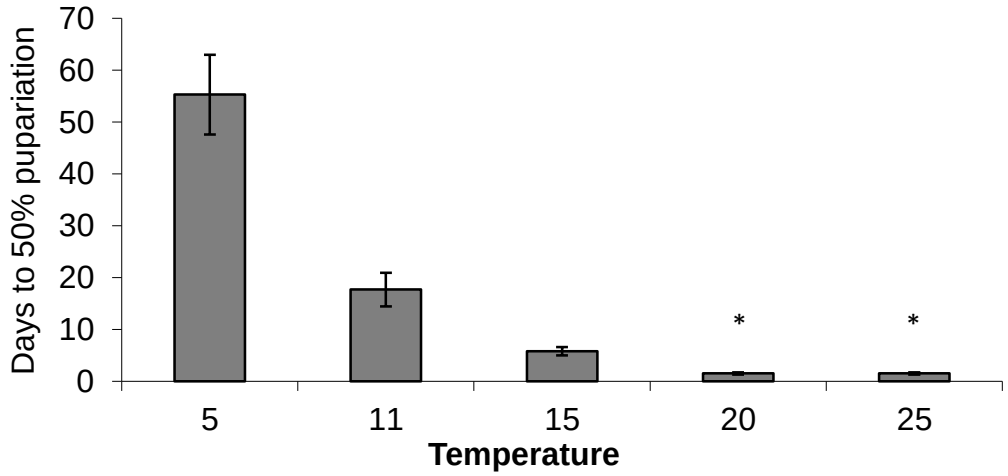


Figure 5.11. Mean (±SE) number of days for 50% of larvae (5 replicates of n=10) to pupate at a range of temperatures. Each datum point is based on 5 replicates of groups of 10 larvae. * designated samples which are not significantly different to each other.

5.4.2.2. Duration of exposure at elevated temperatures required to terminate diapause

Eclosion success was high in all experiments (>90%) and all eclosing adults were seen to have normal morphology. Larvae experiencing a higher termination temperature of 25°C showed a marked decrease in duration of diapause when held at an increased temperature for 24h (fig. 5.10a, Mann-Whitney U test, Z=-2.950, P=0.03). When D0 larvae were held at the lower temperature of 15°C, larvae showed a more linear decline, rather than a sharp decline as recorded in other termination procedures. However, a significant difference was recorded in time to 50% pupariation between control samples (continuously held at 11°C) and those held at an increased temperature for 24h (fig. 5.13c, Z=-2.441, P=0.015).

Larvae at D10 of diapause, which experienced the higher temperature of 20°C, terminated diapause more rapidly than those held at 15°C. When larvae were held at the lower

temperature of 15°C, a significant increase in the number of larvae terminating diapause was noted after 2 days (Z=-2.441, P=*0.015*). At 20°C it took just 24h (Z=-3.077, P=0.002, fig. 5.13).

Larval progeny of adults raised at the lower rearing temperature of 15°C were not seen to change their median number of days to 50% pupariation when held at either 20°C (fig. 5.13b, Kruskal-Wallis test, *X*=0.244, *df*=1, P=0.622) or 15°C (fig. 5.14a, *X*= 2.130, *df*=1, P=0.06).

Control samples from D0, held at the elevated temperature of 20°C (fig. 5.12), took almost twice as long to pupate as D10 larvae (16.67 $\pm$ 1.5d, and 10.2 $\pm$ 1.8d respectively, fig. 5.13). D10 larvae require just 24h of increased temperature (20°C) to experience a sharp reduction in the number of days to 50% pupariation (Z=-3.077, P=0.002). Larvae at an earlier stage of development (D0, fig. 5.12b), required 48h at an increased temperature to cause a significant reduction in the number of days to terminate diapause (Z=-2.975, P=0.03).

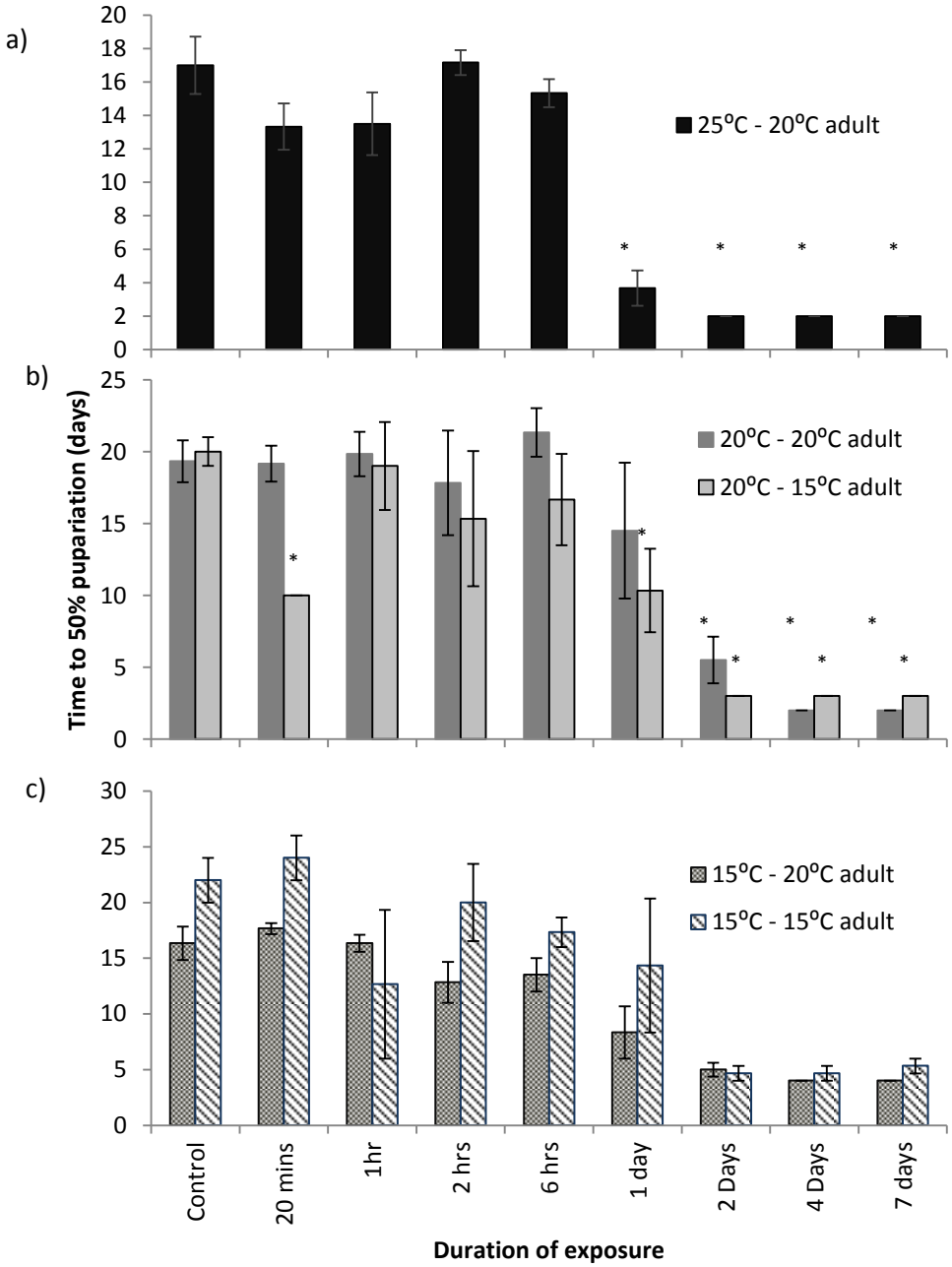


Figure 5.12. The number of days (±SE) that L3 DO *C. vicina* took to reach 50% pupariation, after experiencing a defined period at three different elevated temperatures (n=60 for all data points). a) Adults held at 20°C, subsequent L3 larvae were then held at an elevated temperature of 20°C. b) Adults held at 20°C (dark grey) or 15°C (light grey), subsequent L3 larvae were then held at an elevated temperature of 20°C c) Adults held at 20°C (dotted) or 15°C (dashed), subsequent L3 larvae held at an elevated temperature of 15°C. All larvae were at D0 of diapause when placed at the elevated temperature and were held at 11°C before and after the period of elevated temperature. All adults were placed in a diapause inducing photoperiod (12:12, L:D). Groups labeled with * indicate that the group is significantly different to the control.

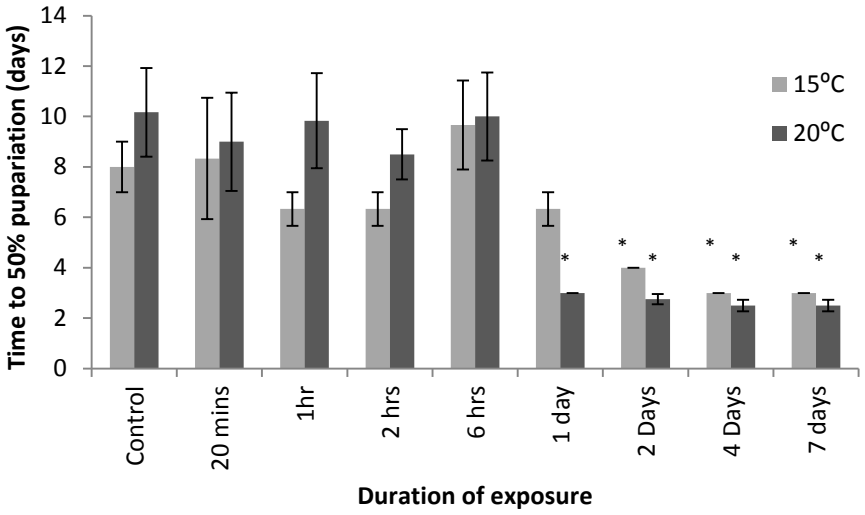


Figure 5.13. The number of days (±SE) that L3 D10 *C. vicina* took to reach 50% pupariation after experiencing a defined period at two different elevated temperatures 15°C (n=30) and 20°C (n=60). Larval progeny of 20°C adults, held at an elevated temperature of 20°C (dark grey) or 15°C (light grey) and subsequently held at 11°C after the period of elevated temperature. Groups labeled with * indicate that the group is significantly different to the control.

5.4.3. Difference between cold tolerance of non-diapause (ND) and diapause (D) larvae: the influence of acclimation, parental thermal history and time spent in diapause

ND larvae held under standard culturing conditions (11°C) had limited tolerance to -7°C, with an LT_{50} of 1.45d (fig. 5.14a); while D larvae cultured at 11°C survived significantly longer, to 12.9d (fig. 5.14b). These were statistically different, as seen by non-overlapping fiducial limits (probit analysis) (Hart *et al.,* 2002; Hatherly *et al.,* 2008; Hughes *et al.,* 2009), which are presented in table 5.3. Acclimating ND larvae to either 0°C or 5°C significantly improved cold tolerance (table 5.3, fig. 5.12.a), with ND acclimated to 5°C having an LT_{50} of 7.6d and ND acclimated to 0°C an LT_{50} of 4.3d. Acclimating D larvae to either 5°C or 0°C also improved cold tolerance (fig. 5.12b) though not significantly (table 5.3).

Although in all but one experiment (D with no acclimation) cold tolerance was seen to increase in larvae from adults reared at a lower temperature (15°C) (fig. 5.12a & b), there were no significant differences recorded between any groups (Table 5.3).

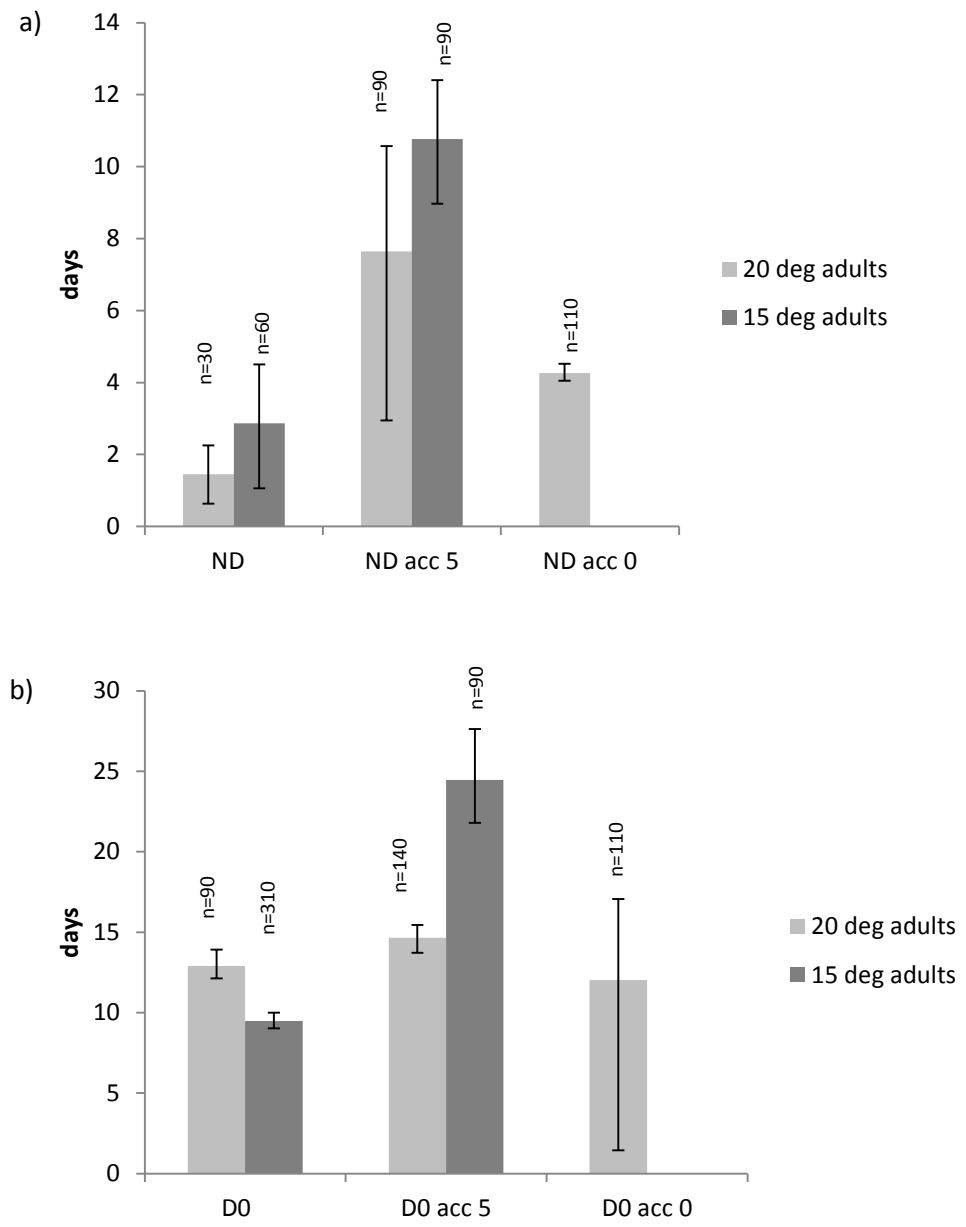


Figure 5.14. LT_{50} (days) (±95% fiducial limits) of (a) non-diapause (ND) and (b) diapause (D) third instar (L3) larvae exposed to -7°C. Larvae were progeny of adults reared at either 20°C (light grey), or 15°C (dark grey) then maintained at either 11, 5 or 0°C for 7 days prior to stress exposure. Exposure of the ND or D larval progeny of 15 degree adults to -7°C was not conducted.

Table 5.3. The upper and lower fiducial limits of LT_{50} at -7°C for ND and D L3 larvae. Larvae were progeny of adults either reared at 20°C or 15°C, termed 'adult treatment'. Two acclimation pre-treatments were also established, 7d at either 5°C or 0°C (termed 'acc 5' and 'acc 0', respectively).

Larval treatment	Adult treatment (°C)	Lower 95% fiducial limit	Upper 95% fiducial limit
DO	20	11.9	13.7
ND	20	0.6	2.3
D0 - acc 5	20	8.0	31.9
ND - acc 5	20	4.7	12.3
D0 -acc 0	20	7.0	22.6
ND - acc 0	20	4.0	4.5
DO	15	9.0	12.7
ND	15	1.1	4.6
D0 - acc 5	15	21.3	27.1
ND - acc 5	15	9.7	11.9
D10	20	3.3	7.2
D20	20	5.7	37.4
D30	20	0.1	3.4
D10	15	3.1	4.3
D20	15	1.9	3.2

As diapause progressed, the cold tolerance of *C. vicina* generally decreased (fig. 5.13), although the decrease was not significant between all of the groups tested (table 5.3). Progeny of adults held at 20°C, D0 larvae were more cold hardy than both D10 and D30 larvae and D20 larvae were more cold hardy than D30 larvae. Progeny of adults held at 15°C, D0 larvae were more cold hardy than D10 and D20 larvae. Adults held at 20°C resulted in larvae exhibiting a greater degree of cold tolerance than those raised at 15°C.

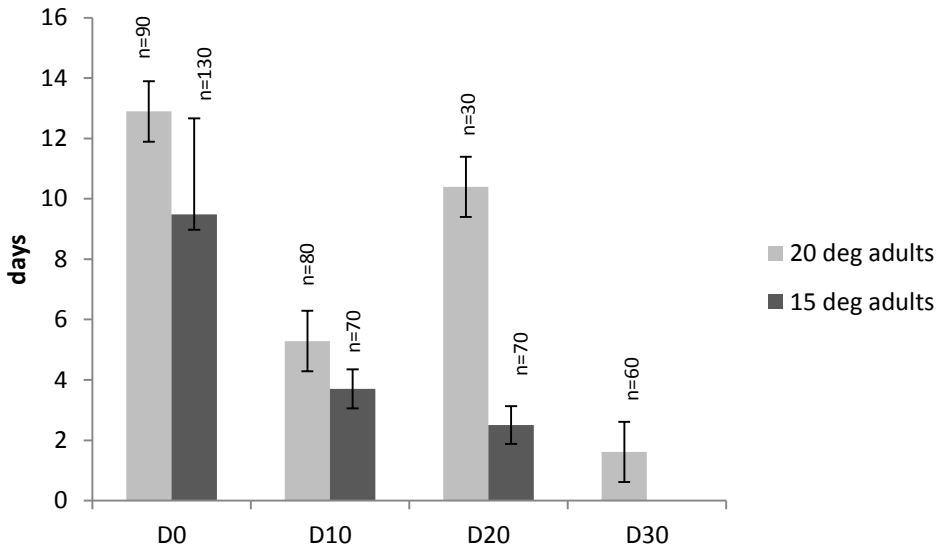


Figure 5.15. LT_{50} (days) (±95% fiducial limits) for diapause (D) third instar (L3) larvae held -7°C at either DO (30d post oviposition), D10 (40d), D20 (50d) or D30 (50d) of diapause. Larvae are progeny of adults raised at either 20°C (light grey), or 15°C (dark grey). The LT_{50} of D30 larvae from 15°C adults was not recorded in this analysis.

5.5. Discussion

Whilst diapause in cold and temperate climates can be affected by many factors, photoperiod and temperature are known to be two key regulators of the process. As a result, this study focuses on the relationship between temperature and photoperiod that is required to allow *C. vicina* to enter and sustain a diapause state under both laboratory conditions and in the field. There are four key findings to this research: firstly, that *C. vicina* eggs oviposited after the 28th October will have reach their CDL. Secondly, temperature is central in the decision to initiate or avert diapause; larvae in a field-simulated global warming scenario (cloche site) show a greatly reduced percentage entering diapause. Thirdly, samples within the laboratory show an increased ability to tolerate cold temperatures if in the diapause state. Finally, diapause can terminate after just 24 hours of exposure to elevated temperature.

An aggregation of adults was noted in both of the October field trials (2009 and 2010). Adult diapause has been recorded in *C. vicina* with adults surviving over 3 months at 6°C (Vinogradova, 2009). Aak *et al*. (2011b) saw adults of *C. vicina* aggregating in late summer at

68°N; they concluded that this was indicative of reproductive diapause. Many other species aggregate whilst in dormancy, such as the Coleopterid rubber litter beetle, *Luprops tristis* (Vinod & Sabu, 2010), and the firebug, *Pyrrhocoris apterus* (Su *et al.,* 2007). Su *et al.,* (2007) investigated the effect of aggregation on metabolism and found that the larger the aggregation, the lower the metabolism; which has distinct advantages for overwintering success. One of the characteristic features of adult reproductive diapause is an arrest of oogenesis in females (Herman, 1981). As such, because adults in the October field trials were still reproductively active throughout this aggregation period, they were not in reproductive diapause. Research has suggested that adults may aggregate for a number of reasons including to increase tolerance to dehydration (Benoit *et al*., 2007a) and as a defensive response against predators (Evans, 2000).

McWatters and Saunders (1996) found that diapause induction is principally initiated by temperature and photoperiod, with a Southern British strain of *C. vicina* having a static photoperiod in the laboratory of 14.5h. One of the principal aims of this study was to examine when the CDL in the field occurred, and hence establish how laboratory designation of CDL corresponds to the field day-length definitions sunrise to sunset, civil twilight (dawn till dusk) or nautical twilight. During the August field trial, adults experienced a photoperiod shorter than the required CDL (if defined as sunrise to sunset) but a photoperiod longer than the CDL defined by civil or nautical twilight. Progeny did not show high levels of diapause in either year at their current overwintering site (open) (fig. 5.4), suggesting that the CDL had not yet been reached. Adults in the September trials would have experienced a photoperiod shorter than either sunrise to sunset or civil twilight; a high percentage of progeny entered diapause in both years (fig. 5.4). This suggests that the CDL in the field matches closely with civil twilight (dawn till dusk), as was proposed by Dalin (2011) who concluded that the leaf beetle, *Phratora vulgatissima,* responded to photoperiod cues correlated with civil twilight. This was further confirmed in this study by the fact that only a very small percentage of larvae from August

photoperiodic controls, entered diapause (6.6 ±1.4%), this was despite larvae experiencing thermal conditions sufficient to induce diapause under normal rearing conditions (11°C in constant darkness). Prior research on *B. coprophila* suggested that sensitivity to light occurred at 6.95 lux (Cloyd *et al*., 2007); with the start of civil twilight having an intensity of c. 3 lux and sunrise c. 400 lux (Johnsen *et al.,* 2006), the model of light sensitivity closely matching to civil twilight is further confirmed.

The temperature at which adults are cultured is also critical in the diapause of *C. vicina,* with McWatters and Saunders (1998) finding that holding adults (from 51°N) at 20°C greatly reduced the incidence of diapause to c.50% from almost 100% in larvae raised at 15°C. During the August field trials, the low incidence of diapause is thought to be mainly due to the CDL not being reached, though temperature did have effects, as indicated by larvae from the August 2010 trial that were held in the laboratory and maintained at 11°C, which saw a 6.6% incidence of diapause. Larvae in the August 2010 trial which were held outdoors and induced a 2.3% incidence of diapause (open site) when mean temperatures were 14.9°C, whereas August 2009 trials saw a 0% incidence of diapause (open site) when mean temperatures were 15.5°C. The CDL had not been reached in any of the August trials but groups of larvae held at progressively lower temperatures did result in a higher incidence of diapause. Maximum temperature was above 20°C in all trials and above 35°C in half of the trials (table 5.1). When only comparing September trials, both of which saw a high incidence of diapause, diapause was highest in 2010 which saw an 8.8°C higher maximum temperature than 2009, but had a mean temperature which was 1.4°C lower. The above suggests that in the field, maximum temperature is a poor key for diapause incidence, whereas mean temperatures appear to play a more central role.

Overall, the percentages of larvae entering diapause in the woodland and open sites were not significantly different (P=0.744), nor were the mean soil temperatures at a depth of 5cm (P=0.94), although the wood site remained more thermally stable, with lower autumnal and

higher winter temperatures. This thermal pattern is probably attributable to the soil structure, water content and wind-breaking effect of woodlands (Baust, 1976). Mean soil temperature at 5cm depth experienced by larvae remained below 15^{o}C at both field sites during all trials (table 5.2), but maximum temperatures well above 15^{o}C were recorded during both August trials (26.2^{o}C in 2009, 18.4^{o}C in 2010). Larvae can avert diapause at temperatures above 15^{o}C (Vaz Nunes and Saunders, 1989), which may account for the low diapause incidence in August field trials, although a similar maximum temperature (19.3^{o}C) was recorded in the September 2009 trial, which had 63% of larvae entering diapause; this suggests that diapause induction is more reliant on the photoperiod experienced by the parental generation than it is on maximum temperature experienced by the larvae themselves.

The cloche site had a significantly lower percentage of larvae entering diapause when compared to both the open and wood sites (P=0.05 and P=0.013 respectively); this is attributable to the cloche site being on average (during diapause initiation) 4.5^{o}C ($\pm$0.07) warmer than the wood site and 4.1^{o}C ($\pm$0.02) warmer than the open site; both temperatures are within the predicted increase by 2100 (1.1–6.4^{o}C; IPCC, 2007).

A cloche was used to simulate global warming conditions; average global surface temperatures have risen by 0.8^{o}C in the last 150 years and are predicted to rise to a maximum of 6.4^{o}C higher by 2100 (IPCC, 2007). Thus the cloche site, with an elevation of 4.5^{o}C, provides an indication of how diapause will be affected if the temperature increases to near the upper limit of current predictions. This 4.5^{o}C temperature elevation was not constant throughout Autumn/Winter, with the greatest difference of soil temperatures at 5cm between the cloche and open/wood site seen in September and decreasing thereafter. The cloche site showed a lower incidence of diapause throughout all of the trials with just 13.2% of larvae entering diapause. Incidence of diapause in the cloche site was highest in the October 2009 field trial in which 42 $\pm$ 3.33% of larvae entered diapause. Diapausing larvae terminated diapause at similar times of the year,

typically three months after it began, e.g. in December 2009 for the September 2009 trial and February 2010 for the October 2009 trial. These data are indicative of an altered phenology, both with regard to the time of entry into diapause and time of diapause termination.

The use of cloches to accurately represent conditions of climate change has been disputed, due to the exclusion or alteration of environmental factors, namely precipitation, U.V. and wind. Bokhorst *et al.* (2011) compared three types of passive warming methods, closed (most like the cloche used in this experiment), ventilated and open top chambers. They found that open top chambers most accurately represented global warming scenarios of all passive warming methods. As the maggots diapause at a depth of 5cm they are unaffected by wind and U.V., precipitation at all three sites was excluded, as such the cloche represents an ideal method of increasing temperature in line with global warming scenarios. Bokhorst *et al.* (2011) noted that all passive warming techniques did not accurately represent conditions as none increased night time temperatures, Godfree *et al*. (2011) overcame this by heating the open top chamber area overnight. The use of soil warming cables or infared heat lamps provide ideal simulated climate change scenarios as they can provide direct control over the temperature at the site. However, the instillation of these with along with the computers, linked thermometers and requirement for constant power render the approach very costly, which was a consideration of this experimental design.

Once diapause is initiated, a generally held view is that a) diapause is terminated due to insects ceasing to respond to diapause maintaining factors; b) there is no direct cue for diapause termination, and c) diapause ends gradually over a period of time (Tauber and Tauber, 1973). The data acquired in this study suggests that diapause termination does not, broadly, end over a lengthy period, but is limited to one month (fig. 5.7b to 5.10b). Experiments conducted under laboratory conditions suggest that diapause larvae are capable of terminating diapause if they experience a relatively short duration (24h) at an increased temperature (≥15^{o}C); this tendency

is exacerbated as larvae age and as the temperature experienced increases. Termination of diapause due to elevated temperatures has previously been seen in *C. vicina,* when it was found that 58% of larvae had pupated after 4 days when held continuously at 25^{o}C (Vinogradova, 1974). In *L. caesar*, pupation began on day 2 or 3 after exposure to 25^{o}C (Ring, 1968). The difference between results in this study and research by Vinogradova (1972) can be explained by the different trigger for recording the end of diapause. In the current study, pupariation was recorded, which was marked by immobilization and subsequent reduction in length due to invagination of segments (Pujol-Luz & Barros-Cordeiro, 2012); Vinogradova, however, recorded the later stage of pupation (which is indicated by eversion of head (Fraenkel & Bhaskaran, 1973)). That said, in the month preceding the peak period for termination of diapause, for instance in January at the open site, during the trial initiated in September 2010, soil temperature at 5cm reached a maximum of 8.0^{o}C (fig. 5.10a), which is well below the temperatures experienced in the laboratory studies. It is known that chilling can decrease diapause duration (Lee & Denlinger, 1996), and this may be the causal factor for the sudden decrease in the number of larvae in diapause, given that larvae experienced a minimum temperature of -0.3^{o}C in January. Alternatively, it could be due to the gradual increase in heat units (accumulated day degrees); although the mean temperature was low (2.9^{o}C), the maximum temperature was 8.0^{o}C, well above even the highest estimate (6^{o}C) for developmental threshold temperature in *C. vicina* (Haskell *et al*., 2000). Larvae would have been accumulating day degrees during the previous (warmer) months and only required a small amount of heat to be absorbed in January to complete diapause. However, there is insufficient evidence in this study to elucidate the precise mechanisms controlling diapause termination in *C. vicina* under field conditions.

Larval viability was high in all but one field trial, October 2010. In this trial, viability at the cloche site was low at just 12.4%, >15% lower than either the open or wood sites. An overwhelming majority of mortality occurred at the egg and L1 stage, when larvae would have been exposed

to ambient air temperature rather than soil temperatures at 5cm. During 2010, air temperatures at ground level reached a minimum of -12.45^{o}C and experienced a mean of -0.64^{o}C. *Calliphora vicina* are thought to have a developmental threshold between 1 and 6^{o}C (Vinogradova & Marchenko, 1984; Greenberg, 1991; Haskell *et al*., 2000; Donovan *et al,* 2006), thus, for a significant proportion of the month, larvae would be too cold to develop. In addition, the mean SCP of diapause-destined L2 *C. vicina* is -14.3^{o}C (chapter 2). Twenty five percent of tested L2 larvae had an SCP higher than -12.45^{o}C; given that *C. vicina* are freeze intolerant (chapter 3), it would be expected to see a 25% mortality in the field samples, due to freeze mortality alone.

The majority of larvae in the cloche site did not enter diapause over all of the trials (86.8% $\pm$2.7%); as such, non-diapause larvae would have been exposed to low temperatures. The LT_{50} of L3 *C. vicina* at -7^{o}C suggested that D larvae are more cold tolerant than ND larvae, with D larvae having an LT_{50} at -7^{o}C of 9.49d and ND larvae just 2.86d; determined for larvae that were the progeny of adults raised at 15^{o}C. Acclimation at 5^{o}C further increased cold tolerance, resultant larvae having an LT_{50} at -7^{o}C of 24.5 days. An increase in cold tolerance while in diapause is not uncommon, having been seen in *T. urticae* (Khodayari *et al*., 2012) and *D. tabulaeformis* (Han *et al*., 2005). By holding adults at alternative temperatures (15^{o}C or 20^{o}C), the biological memory of thermal gradients was assessed by determining the cold tolerance of both diapause and non-diapause progeny. Cold tolerance in ND larvae was seen to be consistently increased in adults that were held at the lower rearing temperature (15^{o}C), though not significantly. Cold tolerance in D larvae, however, resulted in an ambiguous trend, with non-acclimated larvae being significantly more cold tolerant when adults were held at a higher temperature and acclimated larvae more cold tolerant when adults were raised at a lower rearing temperature. These results suggest that *C. vicina* has the capacity to alter cold tolerance in progeny, dependent on not only photoperiodic experience, but also thermal. This decrease in cold tolerance in non-diapause insects could have significant effects for larvae which, due to climate warming, averted diapause despite experiencing their CDL (as was seen in September field trials). Although temperatures

may initially allow direct development, there may come a point when temperatures fall below the necessary level, at which time the insect will cease to develop and remain at that stage until conditions are once again favourable. The stage at which development ceases may not be the stage at which cold tolerance is highest; due to this, the insect may suffer an increased mortality due to the effects of climate change.

The experimental procedure used in this study was designed to look at the effect of increased temperature on phenology, variables such as precipitation, U.V. and wind were all excluded. A passive warming method (cloche) was used to simulate temperatures thought to be induced by global warming. As a passive technique was used, only higher temperatures could be observed, to get a more holistic view of how all aspects of climate change may affect *C. vicina* (an insects) a design which artificially controls temperature and precipitation both during the day and night could be used in future research.

Although climate warming may negatively affect some species, *C. vicina* is likely to be resilient to due to its ability to rapidly cold harden (discussed in chapter 2) and to increase cold tolerance both due to acclimation of larvae and due to lower temperatures experienced by the maternal generation. These reasons may have allowed the majority of *C. vicina* in the cloche site (global warming scenario) in this study to overwinter with a very low incidence of diapause, but with, generally, a very high survival rate. An increase in survival of cold temperatures (either through RCH or acclimation) is thought, partly, to be due to an alteration of metabolites within an organism. These metabolites are thought to differ between larvae in diapause and non-diapause, with larvae in diapause having an increased number of cryoprotectants present. These cryoprotectants are thought to increase overwinter survival as they can protect from the stresses the organism may encounter (including desiccation and cold). A comprehensive, non-biased, comparison of the metabolites present in diapause and non-diapause *C. vicina* has not yet been attempted; as such this is rectified in the following chapter.

6. Metabolomic characterisation of diapause in the blow fly, *Calliphora vicina*

6.1. Summary

The metabolic profiles of diapause and non-diapause third instar larvae were analysed using ^{1}H NMR. Analysis showed a very clear separation of diapause and non-diapause samples. Overall 34 metabolites were identified, 12 metabolites were up-regulated in diapause and 22 metabolites were up-regulated in non-diapause larvae. Analysis of metabolites revealed three main differences within samples:

- The most up-regulated metabolite in diapause samples of *C. vicina* was glycerophosphocholine, which is thought to help alter the fluidity of the phospholipid membrane to prevent damage at low temperatures.
- Energy synthesis in *C. vicina* was found to be different between diapause and non-diapause larvae, with diapause larvae experiencing a decrease in the Krebs cycle, and an increase in the glycolytic cycle. An up-regulation of glucose in diapause samples suggests that diapause larvae have an active glycolytic cycle, and an increase of alanine and subsequent down-regulation of lactic acid suggests they possibly prevent long term build-up of the potential toxic agent, lactic acid, by diverting anaerobic metabolism to produce alanine instead.
- This production of alanine also potentially serves to enhance cold tolerance. A number of other putative cryoprotectants were recorded in *C. vicina* D including glycerophosphocholine, glucose and proline.

This diversity of metabolites indicates a considerable complexity in the mechanisms that underpin the diapause strategy in *C. vicina*; a comparison of data within this study to other studies reveals that species have evolved quite different metabolic strategies to cope with winter cold.

6.2. Introduction

At temperate latitudes, the changing seasons often herald dramatic changes in temperature, rainfall and resource availability. Winter often poses the greatest stress to animals, as low temperatures force the decision to either migrate to warmer latitudes or to stay and endure the season. For species which remain, modification of their behaviour and/or physiology must occur if they are to survive. Many mammals (e.g. the 13-lined squirrel, Serkova *et al*., 2007 and the golden spiny mouse, Levy *et al*., 2012), amphibians (e.g. the water frog, Feidantsis *et al*., 2012) and reptiles (e.g. fresh water turtles, Storey, 2007) either hibernate or enter torpor, while insects go into diapause (e.g. the fall webworm, Li *et al*. 2001).

Metabolomics has recently emerged as a powerful approach for characterising the responses of animals to a range of stressors, including physiological responses (Andrews *et al*, 2009; Bayley *et al*, 2010), toxic insult (Bundy *et al*, 2001; Ellis *et* al. 2011; Poynton *et al,* 2011) and disease (Kang *et al,* 2011; Klein *et al*, 2011). Low metabolic-weight metabolites are the by-products of metabolism and include lipids, carbohydrates and amino acids, amongst other compounds. These metabolites can be analysed using a range of technologies including gas chromatography mass spectrometry (GC-MS), liquid chromatography mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR). ^{1}H-NMR has many advantages over older techniques, such as thin-layer chromatography or high-performance liquid chromatography, as it offers simple sample preparation, high-throughput analysis without chromatographic separation (unlike GC-MS), as well as high reproducibility. Also, all metabolites that contain a non-exchangeable hydrogen atom are impartially assessed during one analysis rather than by assessing individual chosen compounds (Viant, 2008).

In recent years, an increasing number of metabolomic studies have been undertaken to investigate the different cold stress responses of insects, including rapid cold hardening (RCH) (Michaud & Denlinger, 2007; Overgaard *et al*., 2007; Teets *et al*., 2012), cold tolerance (Michaud

et al., 2008, Hawes *et al*., 2008; Kostal *et al.,* 2012), cold-shock (Pedersen *et al*., 2008), and five studies examined the effect of diapause on the metabolome of an insect (Michaud & Denlinger, 2007; Kostal *et al.,* 2011a; Colinet *et al*., 2012a; Xu *et al*., 2012; Zhang *et al*., 2012). Of the studies above, three were performed using NMR (Overgaard *et al*., 2007; Hawes *et al*., 2008; Pedersen *et al*., 2008), the remainder were performed using CG-MS (Michaud *et al*., 2008; Kostal *et al.,* 2011 a, b; Colinet *et al*., 2012a; Kostal *et al.,* 2012; Teets *et al*., 2012). To date, five studies have examined the effect of diapause on the metabolome of insects, all of which used GC-MS as a profiling technique. These were a study of the flesh fly, *Sarcophaga crassipalpis* (Michaud & Denlinger, 2007), the drosophilid fly, *Chymomyza costata* (Kostal *et al*., 2011b), the parasitic wasp, *Praon volucre* (Colinet *et al*., 2012a) and two on the cotton bollworm, *Helicoverpa armigera* (Xu *et al*., 2012; Zhang *et al*., 2012). Michaud & Denlinger (2007) identified elevated concentrations of glycerol, glucose, alanine and leucine in *S. crassipalpis,* and suggested that these might play a role in the up-regulation of glycolysis and cold-tolerance mechanisms. Conversely, a reduction in fumarate, isocitrate, sorbitol and 5 amino acids were also found, which were thought to indicate a reduction in the Krebs cycle. Kostal *et al*. (2011b) identified proline as the most up-regulated metabolite in *C. costata* and subsequently found that diapause larvae supplemented with proline were capable of surviving in liquid nitrogen, once cold acclimated, due to proline assisting in the vitrification of water, which was thought to have prevented cryoinjury. Although Kostal *et al*. (2011b) did identify other metabolites which were up-regulated (glutamine and citric acid) and down-regulated (lactic acid, glutamic acid and alanine) in diapause, no discussion of these metabolites was entered. Glucose and glutamate were the two metabolites most up-regulated in diapause-destined larvae of *H. armigera*, and trehalose was the only metabolite which increased during diapause. It was suggested that trehalose is up-regulated to enhance membrane structure at low temperatures; glutamic acid has been indicated to be important in the long-term storage of memories and its increase was thought to be involved in the storage of diapause-induced photoperiodic information, while

glucose was speculated to have a role in "special memory processes" that are involved during diapause induction (Zhang *et al*., 2012). Other metabolites known to be involved in cold hardiness in diapause are ribitol and sorbitol, not only were they seen to be up-regulated within diapause, but when a mixture of these metabolites were injected into diapausing adults of the red firebug, *Pyrrhocoris apterus,* larvae were three times more likely to survive a treatment at -14^{o}C (Kostal *et al*., 2001).

The blow fly *Calliphora vicina* (Robineau-Desvoidy) is commonly used as a model for investigations of insect diapause (Saunders & Cymborowski, 2003) and cold hardiness (Saunders & Hayward, 1998), due to its distinct diapause phenotype and associated enhanced stress tolerance, as well as ease of culturing. This species has a maternally-regulated facultative diapause as a third instar larva (Vinogradova & Zinovjev, 1972; Saunders, 1987), and in common with most temperate insects, the dominant cues inducing diapause are photoperiod (McWatters & Saunders, 1996) and temperature (Vinogradova & Zinovjev, 1972). *Calliphora vicina* is found from the sub-Arctic (Lebouvier *et al.*, 2011) to the sub-Antarctic (Convey *et al*., 2010) and is a freeze-avoiding species (Block *et al*., 1988). Diapausing larvae have an $LTime_{50}$ of just 12.9 days (d) at -7^{o}C (see chapter 5) making this species less cold-tolerant than *S. crassipalpis*, in which diapause pupae can readily survive 25d at -10^{o}C (Adedokun & Denlinger, 1984). Developmental arrest during diapause is also sensitive to elevated temperatures in *C. vicina*, with U.K. populations averting diapause at temperatures above 15^{o}C (Saunders & Hayward, 1998). In contrast, *S. crassipalpis* sustains diapause/ cell cycle arrest at temperatures of 25^{o}C (Denlinger, 1971). A metabolomics comparison of these and other species will provide a greater understanding of the molecular mechanisms underpinning both diapause regulation and enhanced stress tolerance.

The current study uses ^{1}H NMR to characterise the metabolic fingerprint of diapause in *C. vicina* in order to compare with other species investigated to date. In particular, the intent of this

study is to determine whether metabolic changes associated with diapause are common across species, especially those that play a role in the enhanced cold stress tolerance phenotype. In addition, because metabolomic approaches permit an unbiased assessment (i.e. specific metabolites are not chosen to be assessed) of the diapause phenotype, this study hopes to further identify other mechanisms underpinning dormancy in insects that may have not yet been characterized.

6.3. Method

6.3.1. Culturing of C. vicina

Calliphora vicina cultures were established from field-caught adults collected using bottle traps based on the design of Hwang & Turner (2005), at the University of Birmingham (52°27'N). Cultures were frequently supplemented with freshly caught adults to avoid inbreeding. Adult flies were held under controlled laboratory conditions at 20°C; non-diapause (ND) adults were held under an 18:6 light/dark cycle, diapause (D) adults were held under a 12:12 light/dark cycle. Approximately 200 adults were housed in cages (30cm sided box), with water and sugar provided *ad libtum.* Pieces of liver (15g) were provided for 24 h on days 4, 6, 8 and every day thereafter as a protein source to enable ovary development and as a site for oviposition. On the day of mass oviposition, the eggs were placed in an air-tight container in constant darkness at 20°C for 24 hours to aid hatching, before being transferred to an excess of liver at 11°C. ND larvae were collected on the day of mass wandering, which was day 18 post-oviposition. D larvae were collected on day 30 post-oviposition, which is recognised as the start of diapause (Richard & Saunders, 1987).

Twenty larvae from each of the conditions (D and ND) were used for metabolic analysis. An additional three samples per treatment group were used as technical replicates to ascertain whether variation in the data was due to human errors in sample preparation. All larvae

collected were individually snap frozen in liquid nitrogen and stored at -80^{o}C until extraction was performed.

6.3.2. Preparation of Larvae Extracts

Metabolites were extracted from larvae using a two-step method with a final solvent ratio of 2:2:1.8 (methanol/chloroform/water) (Wu *et al.,* 2008). The polar (upper) fraction was isolated and stored at -80°C for subsequent analysis. The non-polar (lower) phase was isolated, placed in liquid nitrogen for ten seconds and then dried for one hour using a Speed Vac Concentrator (Thermo Savant, Holbrook, NY) and then stored at -80°C. The non-polar extracts were not analyzed in this study.

For the extraction of technical replicates, 3 ND larvae and 3 D larvae were used. After the first homogenization, the 6 samples were pooled and thoroughly mixed. 6 equal aliquots of the sample were then pipetted into 6 glass vials and the reminder of the procedure was carried out as protocol.

6.3.3. ^{1}H NMR spectroscopy

Each polar fraction aliquot was re-suspended in 650 µl of sodium phosphate buffer solution (0.1M, 90% H_2O/10% D_2O, pH 7.0) containing an internal chemical shift standard of 1 mM sodium 3-trimethylsilyl-2,2,3,3-d4-propionate (TMSP). Samples were thoroughly mixed to achieve complete dissolution. Next, samples were centrifuged for 10 mins at 21,885-g, and 600 µl of each supernatant was transferred to a 5 mm outside diameter NMR tube (Norell Inc, Landisville, USA). All tubes were capped and kept refrigerated at ca. 4°C until analysis.

Samples were analysed using a Bruker Avance 500 MHz NMR spectrometer, operating at 500.18 MHz ^{1}H resonance frequency and equipped with a 5 mm cryoprobe (Bruker Biospin, Coventry, UK). Sample temperature was 298K and 5 min was allowed for post-insertion temperature equilibration. Samples were analyzed in a random order.

1-D ^{1}H NMR spectra were acquired using the Bruker pulse sequence 'zgesgp', which provides water suppression by excitation sculpting, modified to include pre-saturation of the water resonance during the relaxation delay. Pulse durations were optimised for the first sample of the batch, with the 90° pulse duration calibrated at 9.38 µs. Other acquisition parameters include a spectral width of 6.01 kHz, relaxation delay of 3s and 96 transients collected into 32,768 data points, with an acquisition time of 10min. Datasets were zero-filled to 65,536 points, and 0.3Hz exponential line-broadening applied before Fourier transformation. All spectra were phased manually, baseline-corrected using a low frequency filter to remove the residual water signal, and calibrated (TMSP at 0.0 ppm) using TopSpin (version 3.0, Bruker).

2-D JRES NMR spectra were acquired using the Bruker pulse sequence 'jresesgp', modified to include pre-saturation of the water resonance during the relaxation delay. Pulse durations were the same as for the 1-D experiments. Other acquisition parameters include spectral widths of 6.01kHz (direct dimension, F2) and 50Hz (indirect dimension, F1), and 8 transients of 16384 data points collected at 32 increments in F1. Zero-filling to 128 data points was applied along F1 only. Prior to Fourier transformation, the SEM window function (Tiziani *et al*., 2008) was applied along F2 and the 0.3Hz sine window function was applied along F1. 2-D spectra were tilted by 45^{o} and symmetrised along F2, as is described elsewhere (Ludwig *et al*., 2010). Skyline projections and pJRES were calculated along F2. Spectra were calibrated (TMSP at 0.0 ppm), with all processing performed using TopSpin (version 3.0, Bruker).

The running of samples and data aquisition as described throughout section 6.3.3. was performed by members of the Birmingham NERC NMR facility.

6.3.4. Pre-processing of pJRES spectra

The pJRES spectra were further processed prior to multivariate data analysis, using the ProMetab software (Viant, personal communication), running within MATLAB (Version 7.0, Mathworks, Cambridge, UK). Spectra were baseline corrected, and regions containing known unwanted signals (reagents used in sample extraction) were excluded. These regions contained signals from methanol (3.349 - 3.38ppm), water (4.72 - 5ppm) and chloroform (7.6-7.8 ppm). Spectra were normalised to give total spectral areas of unity. Spectra were binned with a bin width of 0.005ppm (2.5Hz) between 0.5 and 10ppm. A 'glog' transformation (Parsons *et al*., 2007) to increase the weighting of smaller peaks was performed with a λ value of 9.627e-9 obtained from an optimization using the technical replicates. These technical replicates were pre-processed in precisely the same way as the main data set, to ensure the applicability of the λ value.

6.3.5. Statistical analysis

An unsupervised-model principal components analysis (PCA) of the pre-processed NMR data was generated to show broad-scale variation between the diapause and non-diapause groups, using all the metabolites simultaneously; the PCA was generated using PLS_Toolbox (Version 3.5, Eigenvector Research, Manson, WA, USA) within MATLAB. Each variable within the matrix was mean-centred, but no adjustment was made to equalize the variance of each chemical shift bin due to the glog transformation having been applied previously.

To test for significant differences between individual metabolites within the test parameters (diapause and non-diapause), an ANOVA was run using R (R development Core Team) on the 1800 bins of the 40 biological replicates. Due to the data set being large, the cut-off P-values were adjusted to avoid false positives ($P>0.0001$, false discovery rate (FDR)<0.02%).

6.3.6. Identification of metabolites

The metabolic identity of each significant bin was assessed by comparing pJRES data (in MATLAB) with a metabolite fingerprint library, which had been previously generated by the Birmingham NERC NMR facility. Using software written in MATLAB, potential metabolite matches were identified and conformation of identity was conducted using TopSpin (Bruker Biospin, Fremont, CA)

6.4. Results

A representative spectrum of a proportion of the spectra, showing the mean concentration per bin for D and ND groups is shown in fig. 6.1; the identity of a number of well-resolved signatures of metabolites have been assigned. Technical repeats showed high reproducibility, allowing a high degree of certainty that differences in metabolites were due to differences within samples, rather than errors in sample preparation.

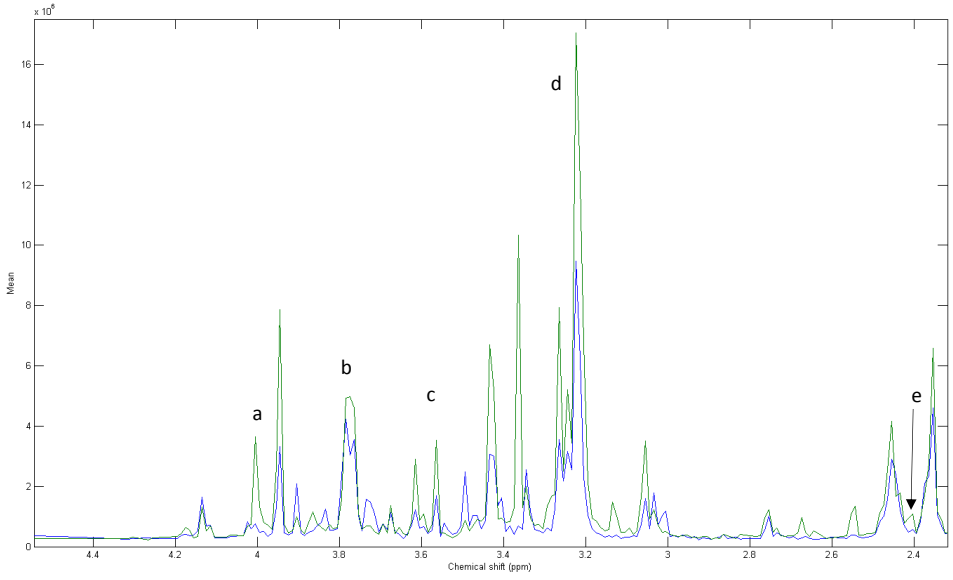


Figure 6.1 Sample spectra from 2.3 to 4.6ppm. Spectra has been subjected to zero-filling, line broadening, Fourier transformation, phase correction, chemical shift referencing, removal of unwanted regions, normalization of total spectral area and then binning to 0.005 ppm. Mean values of all non-diapause samples are shown in green, mean values of diapause samples (excluding sample 17) are shown in blue. Examples of metabolite fractions a- phenylalanine, b-glutamine, c-glycine, d- betaine, e- succinic acid

6.4.1. Multivariate analysis

After pre-processing, PCA plots were generated of the 40 pJRES spectra to show broad-scale differences between the D and ND larvae. Of the 40 samples, sample 17 from the D group was found to be a clear outlier (fig. 6.2), and as such was discarded from all subsequent analysis. Once sample 17 was removed, a high level of consistency was found between peaks of polar metabolites of both D and ND larvae. There was unambiguous clustering of D and ND groups along the first principal component, designated 'PC1' (fig. 6.3a), with PC1 explaining 41.46% of the variance in the data set. The accompanying loadings vector (fig. 6.3b) shows that a wide range of metabolites account for the variance in PC1, with positive peaks corresponding to parts of the metabolic profile up-regulated in ND larvae and negative peaks corresponding to parts which are up-regulated in D larvae. The larger the peak, the greater the involvement with PC1. This large-scale difference along the PC1 axis suggests that, metabolically, D larvae are distinctly different to ND larvae.

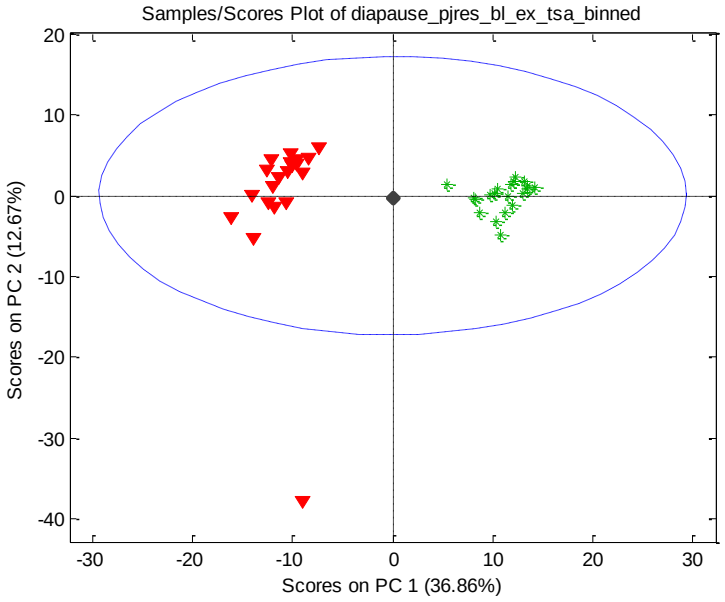
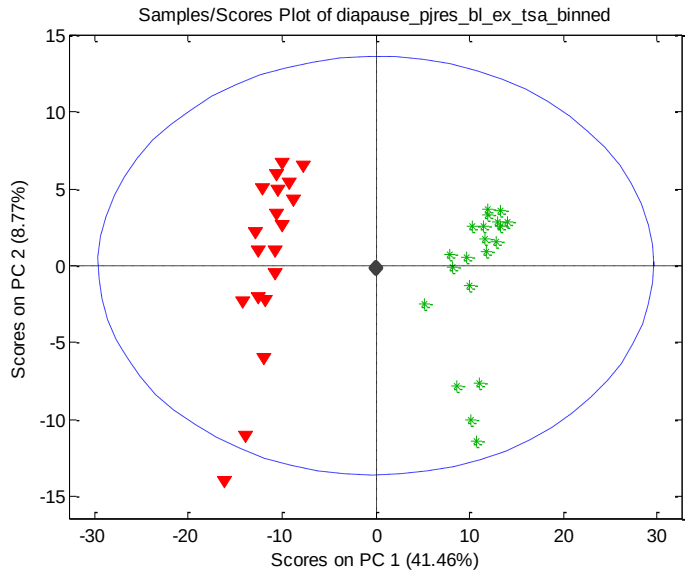


Figure 6.2 PC1/PC2 scores plot for PCA of *C. vicina* larvae pJRES spectra with D samples (red triangles) and ND samples (green star). D sample #17 can be seen as a clear outlier. Technical replicates are shown as a grey diamond.

a)



b)

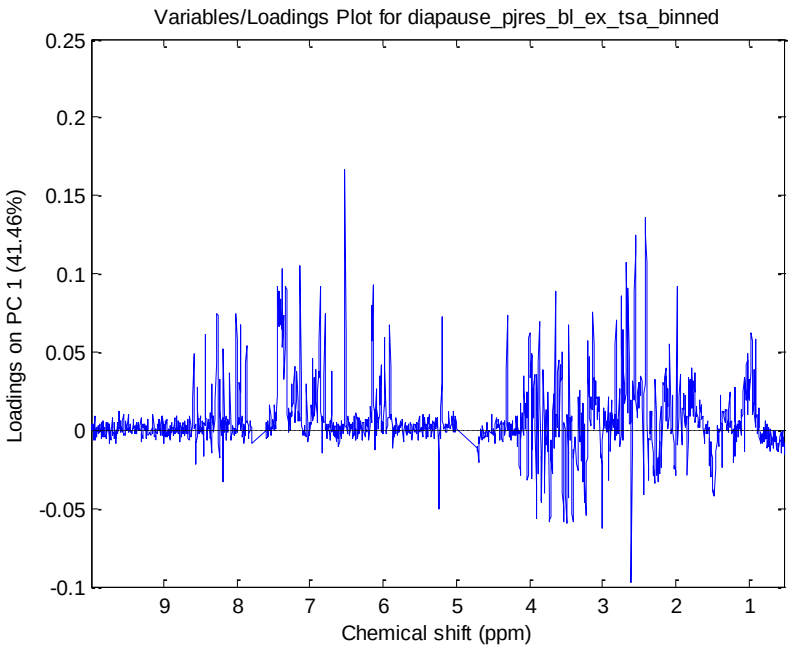


Figure 6.3 a) Repetition of pJRES3 with the exclusion of sample D17. The PC1/PC2 scores plot for the PCA of the blowfly larvae pJRES(3) spectra with diapause samples (red triangles) and non-diapause samples (green diamonds). Figure 6.3b) shows the loadings plot illustrating key peaks leading to the differences noted in PC1. Technical replicates are shown as a grey diamond.

6.4.2. Variation of individual metabolites

In total, 34 metabolites were identified as being up-regulated in either D or ND samples (fig. 6.4); 24 of the 34 metabolites were found to be significantly different between the two groups. The identity of the metabolic profile (Viant, 2008) was first analysed in MATLAB by prewritten automated software and then double-checked in TopSpin by hand; thus identification of metabolites is made with certainty. Only one metabolite, glycerol, could not be allocated an accurate fold change and p-value; this was due its identifiable peaks (at 3.79, 3.66 and 3.57ppm) being shared with other metabolite fractions at the same chemical shifts, although visual analysis of the data clearly suggested that glycerol was increased in ND larvae.

Ten of the twelve metabolites that were up-regulated in diapause larvae (fig. 6.4) were also present in non-diapause larvae: glycerophosphocholine, glucose, α-alanine, pyruvic acid, glycerol-6-phosphate, oxoglutatic acid, phosphocholine, proline, inosine and glutamic acid. Lysine and asparaginine were only found in D larvae. Twelve of the 22 metabolites that were up-regulated in non-diapause were also present in diapause larvae: fumaric acid, phenylalanine, hydroxyglutaric acid, glycine, valine, acetic acid, isoleucine, glutamine, taurine, lactic acid, tyrosine and arginine. The remaining ten metabolites were only present in the non-diapause larvae, which were arginsuccinic acid, malic acid, succinic acid, trehalose, β-alanine, leucine, serine, betaine, threonine and glycerol.

The fold change of the metabolites was established by dividing the peak area of the D samples by the peak area of ND samples, to establish a positive fold change for D samples; the opposite was applied for fold change in ND samples, as was used by Southam *et al*. (2011) and Colinet *et al*. (2012b). Of the twelve metabolites found in D samples, eight were found to be significantly up-regulated in D, while 16 of the 22 in the ND metabolites were found to be significantly up-regulated in ND.

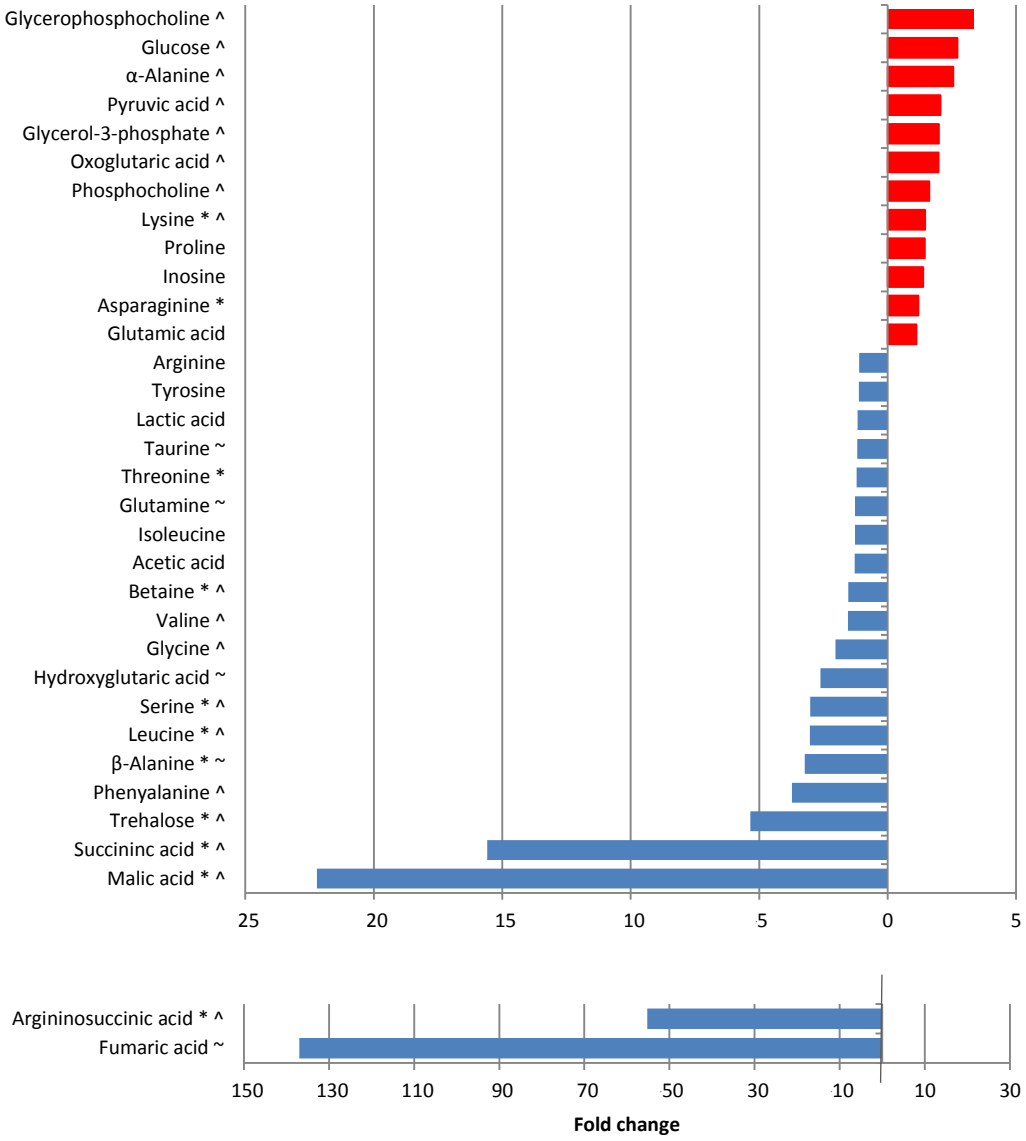


Figure 6.4 Comparison of the 34 metabolites identified in 20 non-diapause or 19 diapause *C. vicina* L3 larval samples after ^{1}H NMR analysis. Quotients of mean content of diapause (D) over non-diapause (ND) were plotted (i.e. fold change). Red bars represent metabolites that were more abundant in D larvae; blue bars represent metabolites more abundant in ND. * designates metabolites only seen in one condition (D or ND). ^ designates metabolite significant to P<0.001. ~ designates metabolite significant to P<0.05

6.5. Discussion

This is the first time that metabolomic characterisation of diapause has been performed using ^{1}H NMR. ^{1}H NMR has proved to be a powerful hypothesis-building technique that has the advantage of performing a non-biased screening of the data against a large library of metabolites, allowing quick detection of the metabolites that characterise a given phenotype. ^{1}H NMR has a wide range of uses from the Identification of biomarkers in human brain tumours (Wright *et al.,* 2010), the characterisation of nicotine stress in Pseudomonas (Ye *et al*., 2012), the characterisation of insect phenotypes such as rapid cold hardening and cold shock in *Drosophila melanogaster* (Overgaard *et al*., 2007) and the characterisation of thermal stress in *D. melanogaster* (Pedersen *et al*., 2008). The results presented in this study show clear separation in the metabolic response found between D and ND larvae, indicating that these two pathways are metabolically distinct. The 34 metabolites recorded here significantly expand the list of metabolites identified in *C. vicina* to date, beyond the five metabolites measured in ND larvae by Block *et al*. (1990) using gas chromatography. The number of metabolites identified in this study was higher than other insect studies using NMR (Overgaard *et al,*. 2007 – 31 metabolites; Pederson *et al*., 2008 - 3 metabolites) but comparable or less than studies which used GC-MS (Michaud & Denlinger, 2007 – 62 metabolites; Kostal *et al*. 2011b – 34 metabolites; Zhang *et al.* 2012 – 49 metabolites).

There are four main advantages of NMR over GC-MS, those being the minimal requirement for sample preparation, the non-discriminating and non-destructive nature of the technique and the potential for high throughput fingerprinting. The disadvantages, in the context of this research are discussed below. One of the dominant metabolites identified in ND *C. vicina* by Block *et al*. (1990) was glucose (5.8µg mol^{-1}), which was seen to increase in D larvae significantly in this study (fig. 6.4). Fructose, sorbitol and myo-inositol were also identified by Block *et al*. (1990), but these compounds were not identified in the current study despite being in the

spectral reference library. The reasons for this are not clear, but could be that ^{1}H NMR is a less sensitive technique than gas chromatography (Viant, 2008). Another metabolite identified by Block *et al*., (1990) was mannitol, and this may not have been identified here because it was not in the spectral reference library. This highlights one of the limitations of ^{1}H NMR in that a positive conformation of metabolite identity can only be made if it can be compared to a reference spectrum of the same metabolite. Despite these limitations, the considerable number of metabolites that have been characterised point towards a number of metabolic processes that are altered within the diapause programme in *C. vicina*.

This research utalised the use of a PCA to visualize the separation between data sets, though this is not the only method by which this can be visualized and quantified. Ramadam *et al.* (2006) found that separation of data was adequate using both PCA and projection to latent structures discriminant analysis (PLS-DA) the separation was dramatically improved if a genetic algorithm was used. A genetic algorithm was not used in this research due to the dramatic separation of data by PCA alone. Recently Worley *et al.* (2013) developed the PCAtoTree software as a method of quantifying and visualizing separation significance in either PCA or PLS-DA score plots, this methodology generates dendrograms from PCA scores, *p* values and bootstrap numbers which would have provided a viable alternative to the methodology used in this study, the only benefit of the PCAtoTree methodology over the methodology used in this study is that it is single-step, as such represents a large saving of time. As such this the PCAtoTree methodology should be considered for future research.

6.5.1. Energy synthesis

The Krebs cycle is used by all aerobic organisms to generate energy. This cycle is known to decrease during diapause (Kageyama & Ohnishi, 1971; Michaud & Denlinger, 2007; Xu *et al*., 2012), and metabolomic characterisation of diapause in *S. crassipalpis* suggests that the shutting down of this cycle results in the accumulation of pyruvic acid and a decrease in fumaric

acid and citric acid (Michaud & Denlinger, 2007). In *H. armigera*, fumaric acid, succinic acid and malic acid were all seen to decrease in diapause pupae (Xu *et al*., 2012) and in *P. volucre* a 1.5 to 2-fold decrease was seen in fumaric acid, malic acid and citric acid (Colinet *et al*., 2012a). In the present study, a much larger change was identified in the Krebs cycle intermediates fumaric acid, malic acid and succinic acid (136.95, 22.22 and 15.58-fold, respectively) in ND larvae. Malic acid and succinic acid levels were reduced to the point that they were not present in D samples at all, representing a complete shutting down of the Krebs cycle in *C. vicina.* Two metabolites from the Krebs cycle were up-regulated in D samples; the Krebs cycle intermediate, oxoglutaric acid, and the Krebs cycle precursor, pyruvic acid. The up-regulation of oxoglutaric acid may be due to its downstream enzyme, α-ketoglutarate dehydrogenase, being suppressed during D; the shutting down of this enzyme would lead to the downstream metabolites (succinic acid, fumaric acid and malic acid) being suppressed in D samples, which was seen in this study. The suppression of α-ketoglutarate dehydrogenase is only speculative and other studies have shown that metabolites upstream were also suppressed (e.g. citric acid - Michaud & Denlinger, 2007; Colinet *et al*., 2012a) as such this may be specific to *C. vicina*.

As was seen in other research (Kukal*,* 1991; Michaud & Denlinger, 2007; Colinet *et al*., 2012a), during diapause, alanine levels increase; this is thought to be due to either a by-product of increased pyruvic acid metabolism or due to a shift to alanine production instead of lactic acid, due to alanine being less toxic to cells (Touchette & Burkholder, 2000). The toxicity theory is supported in this study as an increase in lactic acid was seen in ND samples (1.17 fold change), while an increase in alanine was noted in D samples (2.56 fold increase). Although insects in diapause do experience a decrease in metabolism, a complete shutdown is not thought to occur; instead, there is a growing body of evidence that energy is gained through glycolysis.

In this study, an increased amount of pyruvic acid was also noted in D samples, as was seen by Michaud & Denlinger (2007). Pyruvic acid marks the metabolic link between glycolysis and

aerobic/anaerobic respiration. During glycolysis, glucose is converted to pyruvate; both metabolites were seen to be elevated in diapause pupae of *S. crassipalpis* (30-fold and 6-fold increase respectively; Michaud & Denlinger, 2007). A c. 1.7-fold increase in glucose was seen in diapausing mummies of *P. volucre* (Colinet *et al.,* 2012a) and a 2-fold increase in the elm leaf beetle, *Xanthogaleruca luteola* (Soudi & Moharramipour, 2012). This agrees with the current study, in which a 2.72-fold increase in glucose was seen in D samples. Through a proteomic analysis, a crucial enzyme in glycolysis, glyceraldehyde 3 phosphate dehydrogenase (GAPHD), was seen to be increased in diapause *P. volucre* (Colinet *et al.,* 2012a) and *Nasonia vitripennis* (Wolschin & Gadu, 2009). Glucose is also known to be up-regulated within RCH (Lee *et al.,* 1987; Teets *et al*., 2012); this suggests that an increase in glycolysis may be a common feature of diapause and other stress phenotypes. Through the glycolysis pathway, glucose can be converted to many different polyols and amino acids. Although the increase in glycolysis during overwintering may not be universal to all organisms, given that a decrease in glucose was seen as a key marker for hibernation in the ground squirrel (Serkova *et al*, 2007), which uses glycolysis in summer and switches to fat metabolism in winter, glycolysis may alleviate the risk of an accumulation of toxic lactic acid.

6.5.2. Membrane adaptations

As temperature decreases, the fluidity of membranes decreases; to counteract this, organisms are able to reorganise membranes to increase fluidity. One method of altering membrane fluidity is to alter the ratio of glycerophosphoethanolamines (PE) to glycerophosphocholine (PC) with the ratio of PE to PC increasing at lower temperatures (Hazel, 1989; Hazel, 1995) and during diapause (Tomcala *et al.,* 2006). This increase of PE is thought to be beneficial due to the molecular geometry, with PE having a conical shape and PC have a cylindrical shape; thus PE results in a less well-organised membrane which pack together less efficiently in the laminar phase and increase fluidity. An alternative hypothesis is that PC should increase as temperature

decreases, due to PC having a lower melting point (-20^{o}C) when compared to PE (-16^{o}C). Thus, PC may remain more fluid at lower temperatures. However, it has been suggested that melting temperature is more dependent on the intrinsic chain-melting temperature than the specific headgroup (Wan *et al.,* 2008).

Interestingly, PC showed a 3.37-fold increase in D relative to ND larvae, and was the most up-regulated metabolite during diapause. Although this research has shown an increase in PC during D, PE was not identified in either D or ND; this is due to PE not being available in the reference library. Using the non-polar fraction of the bi-phasic fraction, a lipidomic analysis of the lipid composition for *C. vicina* is currently being undertaken by another member of the laboratory; this will hopefully give a better understanding of any membrane adaptations in *C. vicina.* An increase in the proportion of PE to PC was noted in *S. crassipalpis* in response to diapause but not chilling, using thin-layer chromatography (Michaud & Denlinger, 2006).

6.5.3. Putative cryoprotectants in diapause

The term 'cryoprotectant' typically refers to polyols and sugars, but an increasing number of amino acids are being found to have a role in the protection of insects against various types of stress, and as such have been included as cryoprotectants in this analysis. The number of polyols and sugars up-regulated in diapause within this study was unexpectedly low, with only glucose and glycerol-3-phosphate being increased. The up-regulation of glucose in diapause has previously been found in the European corn borer, *Ostrinia nubilalis,* (Stanic et al. 2004), *S. crassipalpis* (Michaud & Denlinger, 2007), *X. luteola* (Soudi & Moharramipour, 2012) and *P. volucre* (Colinet *et al.,* 2012a). In this study, glucose was elevated 2.73-fold in D compared to ND (fig. 6.4). Glucose, along with other cryoprotective molecules, was found to be implicated in the maintenance of cell functions while insects are at low temperatures (Bale, 2002). Glucose appears to be important for overwintering in other poikilotherms; with a seasonal elevation of

glucose being seen in early winter in the tree frog, *Rana sylvatica*; furthermore, glucose was seen to confer an increased ability of this species to survive freezing (Costanzo & Lee, 2005).

Glycerol-3-phosphate was also significantly up-regulated in *C. vicina* in diapause samples. This metabolite is a precursor molecule in the glycolysis cycle, which sees the breakdown of glucose to pyruvate. Glycerol-3-phosphate is produced from glycerol, and forms the primary backbone of triglycerides and glycerophospholipids; as such, the increase noted in diapause samples may be stores to allow membrane adaptation if cold temperatures occur. Alternatively, glycerol-3-phosphate could be utilised as an energy store either during or post diapause. No studies have described the effect that glycerol-3-phosphate has on cold tolerance in insect species, which may be due to either older techniques not being able to differentiate between glycerol and glycerol-3-phosphate, or due to glycerol-3-phosphate not being found in other studies. Glycerol-3-phosphate has been found in the black-tailed prairie dog, *Cynomys ludivicianus*, in a study in which it was found that the enzymes surrounding glycerol-3-phosphate were favoured at low temperature, due to their increased affinity for hibernator muscles (de la Roche, 2012).

Alanine (α) levels were significantly elevated in *C. vicina* (fig. 6.4); this has been identified as a common characteristic during diapause and has been seen in the European corn borer, *O. nubilalis* (Morgan & Chippendale 1983), the barnyard grass stem borer, *Enosima leucotaeniella* (Goto *et al.,* 1998b), the fall webworm, *Hyphantria cunea* (Li *et al.,* 2001) and in *S. crassipalpis*, which saw a threefold increase (Michaud & Denlinger, 2007). Alanine is not up-regulated in all overwintering animals, however, and again hibernating mammals appear to employ different strategies, with alanine decreasing in the ground squirrel when in hibernation (Serkova *et* al., 2007). Alanine is thought to suppress apoptosis (Franek & Sramkova, 1996) and is thought to contribute to cold hardiness though synergic effects with other solutes (Churchill & Storey, 1989). Evidence suggests that alanine may be playing a role in enhancing stress tolerance, as supplementing larvae with alanine was seen to increase cold tolerance in *C. vicina* larvae

(Coleman, unpublished data). However, this is not universal to all species as in the oriental corn borer, *O. furnacalis*, despite diapause larvae accumulating alanine, no increase in cold hardiness was recorded (Goto *et al*., 2001). In humans, an increase in alanine enhances levels of HSP70 mRNA, when combined with thermal stress (Nissim *et al.,* 1992). Heat-shock proteins play a vital role in protecting organisms from a range of stresses including: thermal stress (Qin *et al*., 2005), desiccation (Mizrahi *et al*., 2010), anoxia (Monari *et al*., 2011) and chemical stressors (Yoshimi *et al*., 2009); they have also been shown to be up-regulated during diapause (Rinehart *et al*., 2007; Hayward *et al*., 2005). Thus, an increase in alanine may enhance stress tolerance in insects by up-regulating HSP's.

Two key metabolites were unexpectedly down-regulated during diapause, trehalose and glycerol. Trehalose is up-regulated in several insects due a variety of stressors including anoxia (Chen *et al*., 2002), desiccation (Benoit *et* al., 2009) and thermal stress (Diamant *et al.,* 2001). Trehalose was seen be increased by 1.56-fold in diapause-destined larvae of *H. armigera* (Zhang *et al*., 2012). Glycerol was seen to be up-regulated during diapause in larvae of *O. furnacalis* (Goto *et al.,* 2001), in which study it was noted that glycerol content was low during early winter (October) but increased in January; as such the increase in glycerol concentration may have been due to the low temperatures recorded at the study sites during January (2^{o}C) rather than as a result of diapause. In the D larvae stored at the non-stressful temperatures in this study (11^{o}C), the response to enhance trehalose levels may not have been elicited. Glycerol levels commonly increase during diapause in other species such as *O. nubilalis* (Stanic *et al.,* 2004) and *S. crassipalpis* (Michaud & Denlinger 2007). In this study, glycerol was only found in non-diapause larvae, although a confirmed p-value or fold change could not be assessed; previous studies on *C. vicina* have revealed only trace amounts if glycerol in non-diapause larvae (Block *et al.,* 1990). Glycerol is a polyol that can protect proteins and membranes, and promote supercooling of body fluids (Bennett *et al.,* 2005); it is thought to act in synergy with heat shock proteins by acting as a chemical chaperone (Diamant *et al.,* 2001).

6.5.4. Conclusion

Energy synthesis in *C. vicina* is notably different in D and ND larvae, with D samples experiencing a shutting down of the Krebs cycle, and apparently switching to the glycolytic cycle instead. This observation is supported by other metabolomic studies of diapause, e.g. in *S. crassipalpis* (Michaud & Denlinger 2007) and the Antarctic midge, *Belgica antarctica* (Michaud *et al*., 2008), and indicates that the metabolic shift may be common in insect overwintering dormancy. An up-regulation of alanine and subsequent down-regulation of lactic acid in D samples suggests that whilst D larvae have an active glycolytic cycle, they prevent long term build-up of the potential toxic agent, lactic acid, by diverting the pathway to produce alanine. This production of alanine also potentially serves to enhance cold tolerance, as evidenced by the role of this metabolite in the cold tolerance in, *O. nubilalis* (Morgan & Chippendale, 1983) and *H. cunea* (Li *et* al., 2001), as well as more recent supplementary feeding studies with *C. vicina* (Coleman, unpublished). A number of other putative cryoprotectants were noted in *C. vicina* D, including glycerophosphocholine and glucose. This differs from the list of primary cryoprotectants identified in other species, e.g.: glucose and trehalose were identified in the cotton bollworm, *Helicoverpa armigera* (Zhang *et al*., 2012); proline and glutamine in the drosophilid fly, *Chymomya costata* (Kostal *et al*., 2011b) and glucose, pyruvate and alanine in *S. crassipalpis* (Michaud *et al*., 2007). This indicates considerable diversity in the mechanism underpinning cold tolerance in diapause, and that species have evolved quite different metabolic strategies to cope with winter cold.

7. General discussion

This thesis concentrates on four key objectives: the effect of two primary stresses (cold and desiccation) on two species of Calliphoridae, *Calliphora vicina* and *C. vomitoria*. How cold temperatures affect diapause in *C. vicina* and try to elucidate if *C. vomitoria* entered diapause. The effect that increased temperatures, due to global warming, may have on the phenology of *C. vicina*. See if diapause and non-diapause larvae of *C. vicina* have a distinct 'metabolomic fingerprint'.

The results of the above objectives are broken down in to two sections: unquestionable new results and speculative new results and are discussed below.

7.1. Unquestionable new results

This appears to be the first research (to the best of knowledge) concerning the optimization of the culturing of *C. vomitoria*. One of the largest differences observed was concerning the effect of rearing *C. vomitoria* in dry conditions. Post-wandering *C. vicina* are commonly reared in dry sawdust with 100% survivorship, this was not seen in *C. vomitoria* in which survival was just 46.6%, this dramatically increased to 96.6% once larvae were held in a medium with an equal weight of sawdust to water. Holding *C. vomitoria* in damp sawdust altered the temperature at which larvae froze, with their SCP increasing by 5^{o}C.

The designation of if a species is freeze tolerant or freeze avoiding is currently determined by observing survivorship after the insect has been taken to its SCP this is generally determined using dry larvae. In this thesis it was noted that if larvae were frozen via ice inoculation a greater proportion survived, particularly *C. vomitoria* who were seen to have a 0% survival if dry larvae were removed at their SCP or 49.1% survival when larvae were inoculated with ice. This 'partial freeze tolerance' has been noted before (Brown *et al*., 2004; Hawes *et al*., 2010) and as such there is a growing body of evidence which suggests that both the SCP of dry and ice inoculated

larvae should be taken before a classification of freeze tolerant/ avoiding should be made. *Calliphora vomitoria* were seen to have the ability to rapidly cold harden, as was noted in *C. vicina* Coleman (unpublished). *Calliphora vomitoria* were seen to have a lower DTemp (-13^{o}C compared to -9.5^{o}C in *C. vicina*) and showed a stronger response after a 2h acclimation at -5^{o}C (77.5% and 66.0% increase respectively) suggesting that *C. vomitoria* have a greater capacity to rapidly cold harden. *Calliphora vicina* that were in diapause were seen to have an increased LT50 if they were not in diapause, with diapause larvae showing 50% mortality after 9.49 days at -7^{o}C and non-diapause just 2.89 days at the same temperature. This was seen to increase in diapause larvae if they were first acclimated at 5^{o}C for 7 days, with 50% mortality not being reached until 24.5 days.

Flies which were newly hatched adult on the 14th of September (or afterwards) were able to produce progeny in which >50% entered diapause. The designation of CDL for *C. vicina* was seen to most closely correlate with civil dawn/dusk. Larvae held under a cloche had a lower rate of diapause than those reared in the woodland or open field; larvae were also seen to have decreased diapause duration.

Through using metabolomics diapause and non-diapause larvae were seen to have very distinct metabolic fingerprints. Furthermore analysis of the metabolites showed that diapause larvae shut down the Krebs cycle and instead rely on energy being gained via the glycolytic cycle. surprisingly only a few cryoprotectants were seen to be present in diapause samples, suggesting that they are produced at the onset of cold temperatures, rather than in anticipation of them.

7.2. Speculative new results

Due to limitations of trap design and permission from local land owners it was not possible to create field fresh cultures of *C. vomitoria* as was done for *C. vicina*; instead insects were bought from a fishing tackle shop in Birmingham. No prior thermal or generational history could be

ascertained. There were a number of species present in the maggots purchased including *C. vicina, C. vomitoria* and at least two *Lucilia* species (not further identified). It was not known if there has been any bottle necks in the breeding before they were bought. Using these flies we were able to create a hybrid of *C. vicina* and *C. vomitoria*, though due to the unknown history of *C. vomitoria* the experiment should be repeated with field fresh insects before this can be confirmed.

Calliphora vicina were significantly more desiccation resistant than *C. vomitoria* with *C. vicina* having 80.5% of initial water content remaining after 10 days of desiccation at 5% RH at 5^{o}C, whereas *C. vomitoria* had just 43.1% remaining. Though as was seen with other studies (Cornette & Kikawada, 2011) high levels of desiccation resistance do not always imply high levels of desiccation tolerance. Despite *C. vicina* having a high level of desiccation resistance, they had lower tolerance levels than *C. vomitoria*. Both *C. vicina* and *C. vomitoria* displayed a cross tolerance (cold and desiccation) phenotype. Although results for desiccation resistance and tolerance were clear, the number of replicates was low throughout all experiments, as such it would be ideal to increase the number of replicates to ensure the results are accurate.

Using a passive warming technique it was found that larvae held under a cloche have a decreased chance of entering diapause than their counterparts not under a cloche suggesting that diapause may be negatively influenced in global warming continues as is predicted (fig 7.1). Although the average temperatures which were recorded in the cloche represent the IPCC maximum predicted thermal rise by 2100, all other variables were excluded. As such to gain a complete view of how all aspects of climate change may effect *C. vicina* a different experimental design is needed, ideally one which employs soil warming tubes that allow for night, as well as day, time increases of temperature. A design that did not exclude precipitation would also be ideal, as moisture in can make a large difference to survivorship, SCP and cold tolerance (chapters 2, 3 and 4). Although the current experimental design did highlight how increased

temperature may affect diapause in the future, a different design is needed if seeking to answer how climate change will affect diapause.

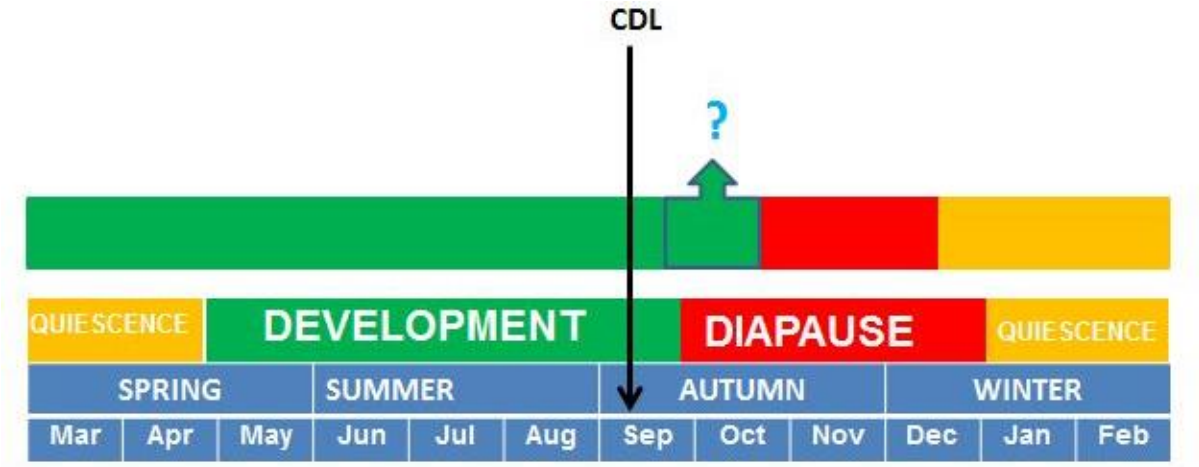


Figure 7.1. Schematic depiction of the effect of climate warming upon diapause. The lower multi-colored line represents current transition through stages of development (green), diapause (red) and post-diapause quiescence (yellow). The thick arrow represents the time of year at which *C. vicina* (at 52°N) currently experiences its critical day length (CDL) (around 5th September). Because diapause initiation in the majority of *C. vicina* can only occur if mean soil temperature remains below 15°C, an increase in temperature may lead to a delay in entrance into diapause or complete diapause aversion. This is represented by the upper multi-coloured line, as is an early termination of diapause and a shorter post-diapause quiescence. Figure modified from Hayward *et al*., unpublished.

Although the number of metabolites that were found through using metabolomics far exceeds research undertaken by Block *et al*. (1990), the determination of the positive identification of any metabolite is done so against a reference library, the reference library contained 210 metabolites at the time of analysis. This is thought to represent just 10% of any organisms metabolome, as such to get a thorough representation of the metabolic fingerprint of diapause a larger reference library is required.

7.3. Future directions

To further the work conducted on the impacts of climate change on insect phenology there are two primary areas which would be interesting. Firstly, a more holistic view of all aspects of

climate change could be undertaken, including U.V., temperature and precipitation. This could be furthered by using metabolomics to elucidate the metabolic profile of *C. vicina* as it progressed through winter. Also, the impact climate warming would have on diapause induction could be observed. In this thesis, only the effects of increased temperatures on the larval stages was observed; by placing adults in a climate warming scenario, a greater understanding of the effects of a warmer climate could be observed. Secondly, a field trial of the overwintering of *C. vomitoria* could be conducted, this would not only elucidate the current overwintering strategy of *C. vomitoria,* but if concurrently conducted with a climate change scenario, it could gather further information on the effect that climate change could have on species which, potentially, do not overwinter in diapause.

The differing cold tolerance strategies of *C. vicina* and *C. vomitoria* were certainly unexpected, as was the large effect that moisture had upon the survival of *C. vomitoria*. The desiccation resistance of an insect is thought to be due to three primary approaches, cuticular, respiratory and refractory. Although the cuticular profiles of *C. vomitoria* and *C. vicina* have been assessed and found to be similar at the third instar stage (Roux *et al.,* 2006), the respiration has not yet been assessed. Methodology described by Benoit *et al*., (2007b) may allow this.

The fact that ♀ *C. vicina* and ♂ *C. vomitoria* were able to produce a hybrid was noteworthy in itself, though the alteration of stress phenotypes seen in the hybrid compared to the parental strains was more so. The use of RNA interference (RNAi) on these species would not doubt help to highlight genes that are involved with stress phenotypes.

Calliphora vicina is able to pass on biological memory of thermal gradients to its progeny, an epigenetic comparison of the DNA methylation and/or histone modification in diapause and non-diapause larvae would be very interesting.

One of the recurring questions throughout this thesis is how such closely related species as *C. vicina* and *C. vomitoria* show such divergent responses to stress. Although there may be other reasons, the effects of one organism keep appearing throughout wider reading of the literature - the bacterial endosymbiont *Wolbachia. Wolbachia* may be able to increase the SCP and freeze tolerance of insect, as it is thought to be capable of acting as an ice nucleating agent (Maes *et al*. 2012). The presence of *Wolbachia* can be analysed through PCR analysis (Turba *et al*., 2012) and is thought to be eliminated through the application of two antibiotics, tetracycline and rifampicin; therefore it would be interesting to determine whether *Wolbachia* was present in *C. vicina* and if a 'partial freeze tolerance' could be achieved if bacteria were removed. Likewise, it would be interesting to observe whether the limited ability to tolerate freezing in *C. vomitoria* could be lost if infected with *Wolbachia*.

The identification of *Wolbachia* in Calliphoridae species would also have effects beyond mere interest. The COI region can be used to determine the identity of forensically important flies (Harvey *et al*., 2008). The identification of species is of fundamental importance as the development rate is different between species, and it is this which is used to determine the post mortem interval (estimated length of time since death occurred). The COI region is known to be affected by *Wolbachia* (Whitworth *et al*., 2007) and as such could lead to erroneous results which may lead to the misidentification of species and the incorrect calculation of post mortem interval.

8. References

Aak, A., Birkemoe, T., Knudsen, G. K. (2011a) Efficient mass trapping: catching the pest, *Calliphora vicina*, (Diptera, Calliphoridae), of Norwegian stockfish production. *Journal of Chemical Ecology*, 37, pp. 924–931.

Aak, A., Birkemoe, T., Leinaas, H. P. (2011b) Phenology and life history of the blowfly *Calliphora vicina* in stockfish production areas. *Entomologia Experimentalis et Applicata*, 139, pp. 35–46.

Addo-Bediako, A., Chown, S. L., Gaston, K. J. (2000) Thermal tolerance, climatic variability and latitude. *Prococeedings for the Royal Society of Biological Sciences,* 267, pp. 739–745.

Adedokun, T. A., Denlinger, D. L. (1984) Cold-hardiness: a component of the diapause syndrome in pupae of the flesh flies, *Sarcophaga crassipalpis* and *S. bullata. Physiological Entomology*. 9, pp. 361-364.

Adhikari, B. N., Wall, D. H., Adams, B. J. (2010) Effect of slow desiccation and freezing on gene transcription and stress survival of an Antarctic nematode. *The Journal of Experimental Biology*, 213, pp. 1803–1812.

Adis, J. & Junk, J. J. (2002) Terrestrial invertebrates inhabiting lowland river floodplains of central Amazonia and central Europe: a review. *Freshwater Biology* 47, pp. 711–731

Ames, C. & Turner, B. (2003) Low temperature episodes in development of blowflies: implications for postmortem interval estimation. *Medical and Veterinary Entomology,* 17, pp. 178-186.

Amir, G., Rubinsky, B., Basheer. S. Y., Horowitz, L. Jonathan, L., Feinberg, M. S., Smolinsky, A. K., Lavee, J. (2005) Improved viability and reduced apoptosis in sub-zero 21-hour preservation of transplanted rat hearts using anti-freeze proteins. *Journal of Heart and Lung Transplantation.* 24, pp. 1915-1929.

Ando, Y. (1993) Thermal response and reversibility of diapause in the eggs of *Locusta migratoria*. *Physiological Entomology*. 18, pp. 1-6.

Aneja, M., Gianfagna, T. J., Hebbar, P. K. (2005) *Trichoderma harzianum* produces nonanoic acid, an inhibitor of spore germination and mycelial growth of two cacao pathogens. *Physiological and Molecular Plant Pathology* 67, pp. 304–307.

Anderson, G. S. (2000) Minimum and maximum development rates of some forensically important Calliphoridae (Diptera). *Journal of Forensic Sciences,* 45, pp. 824-832.

Andrews, M. T., Russeth, K. P., Drewes, L. R., Henry, P. G. (2009) Adaptive mechanisms regulate preferred utilization of ketones in the heart and brain of a hibernating mammal during arousal from torpor. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology,* 296, pp. R383-R393.

Amendt, J., Krettek, R., Zehner, R. (2004) Forensic entomology. *Die Naturwissenschaften*, 91, pp. 51–65.

Atay-Kadiri, Z., Benhsain, N. (2005) The diapause of gypsy moth, *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae). *Annals of the New York Academy of Sciences*. 1040, pp. 219–23.

Bale, J. S. (1987) Insect Cold Hardiness - Freezing and Supercooling - an Ecophysiological Perspective. *Journal of Insect Physiology,* 33, pp. 899-908.

Bale, J. S., Harrington, R., Clough, M. S. (1988) Low-Temperature Mortality of the Peach-Potato Aphid *Myzus-Persicae*. *Ecological Entomology,* 13, pp. 121-129.

Bale, J. S. (1991) Insects at low-temperature - a predictable relationship. *Functional Ecology*, 5, pp. 291-298.

Bale, J. S. (1993) Classes of Insect Cold-Hardiness. *Functional Ecology,* 7, pp. 751-753.

Bale, J. S. (1996) Insect cold hardiness: A matter of life and death. *European Journal of Entomology,* 93, pp. 369-382.

Bale, J. S., Worland, M. R. and Block, W. (2001) Effects of summer frost exposures on the cold tolerance strategy of a sub-Antarctic beetle. *Journal of Insect Physiology*. 47, pp. 1161-1167.

Bale, J. S. (2002) Insects and low temperatures: from molecular biology to distributions and abundance. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences,* 357, pp. 849-861.

Bale, J. S., & Hayward, S. A. L. (2010) Insect overwintering in a changing climate. *The Journal of Experimental Biology*, 213, pp. 980–994.

Baust, J. G. (1976) Temperature buffering in an Antarctic microhabitat. *Annals of the Entomological Society of America*, 69, pp. 117-120.

Baust, J. G. & Nishino, M. (1991) Freezing tolerance in the goldenrod gall fly (*Eurosta solidaginis*). In: *Insects at Low Temperature* (Lee R. E &. Denlinger D. L eds.). Chapman and Hall, New York, pp. 260–275.

Bayley, M. & Holsmtrup, M. (1999) Water vapour absorption in arthropods by accumulation of myoinositol and glucose. *Science* 285, pp. 1909-1911.

Bayley, M., Petersen, S. O., Knigge, T., Kohler, H. R. and Holmstrup, M. (2001) Drought acclimation confers cold tolerance in the soil collembolan *Folsomia candida*. *Journal of Insect Physiology,* 47, pp. 1197-1204.

Bayley, M., Overgaard, J., Hoj, A. S., Malmendal, A., Nielsen, N. C., Holmstrup, M., Wang, T. (2010) Metabolic Changes during Estivation in the Common Earthworm *Aporrectodea caliginosa*. *Physiological and Biochemical Zoology,* 83, pp. 541-550.

Baz, A., Cifrian, B., Diaz-Aranda, L.M., Martin-Vega, D. (2007) The distribution of adult blow-flies (Diptera: Calliphoridae) along an altitudinal gradient in Central Spain. *Annales de la Socirte Entomologique de France*, 43, pp. 289–296.

Bean, D. W., Dudley, T. L., Keller, J. C. (2007a) Seasonal timing of diapause induction limits the effective range of *Diorhabda elongata deserticola* (Coleoptera: Chrysomelidae) as a biological control agent for tamarisk (Tamarix spp.). *Environmental Entomology.* 36, pp. 15–25.

Bean, D. W. T., Wang, R. J., Bartelt, B. W., Zilkowski, R. (2007b) Diapause in the leaf beetle *Diorhabda elongata* (Coleoptera: Chrysomelidae), a biological control agent for tamarisk (Tamarix spp.). *Environmental Entomology.* 36, pp. 531–540.

Beck, S. D. (1980) *Insect Photoperiodism.* 2nd Edition. Academic Press: New York, N.Y., USA.

Bemani, M., Izadi, H., Mahdian, K., Khani, A., Amin Samih, M. (2012) Study on the physiology of diapause, cold hardiness and supercooling point of overwintering pupae of the pistachio fruit hull borer, *Arimania comaroffi*. *Journal of Insect Physiology*, 58, pp. 897–902.

Bennett, V. A., Sformo, T., Walters, K., Toien, O., Jeannet, K., Hochstrasser, R., Pan, Q. F., Serianni, A. S., Barnes, B. M. & Duman, J. G. (2005) Comparative overwintering physiology of Alaska and Indiana populations of the beetle *Cucujus clavipes* (Fabricius): roles of antifreeze proteins, polyols, dehydration and diapause. *Journal of Experimental Biology*, 208, pp. 4467-4477.

Benoit, J. B., Del Grosso, N. A, Yoder, J. A, Denlinger, D. L. (2007a) Resistance to dehydration between bouts of blood feeding in the bed bug, *Cimex lectularius*, is enhanced by water conservation, aggregation, and quiescence. *The American Journal of Tropical Medicine and Hygiene*, 76, pp. 987–993.

Benoit, J. B., Lopez-Martinez, G., Michaud, M. R., Elnitsky, M. A, Lee, R. E., Denlinger, D. L. (2007b) Mechanisms to reduce dehydration stress in larvae of the Antarctic midge, *Belgica antarctica*. *Journal of Insect Physiology*, 53, pp. 656–667.

Benoit, J. B., Elnitsky, M. A., Schulte, G. G., Lee, R. E., Denlinger, D. L. (2008) Antarctic Collembolans Use Chemical Signals to Promote Aggregation and Egg Laying. *Journal of Insect Behavior*, 22, pp. 121–133.

Benoit, J. B., Lopez-Martinez, G., Elnitsky, M. A., Lee, R. E., Denlinger, D. L. (2009) Dehydration-induced cross tolerance of *Belgica antarctica* larvae to cold and heat is facilitated by trehalose accumulation. *Molecular & integrative physiology*, 152, pp. 518–523.

Benoit, J. B. (2010) Water management by dormant insects: comparisons between dehydration resistance during summer aestivation and winter diapause. 49, pp. 209-292.

Benoit, J. B., Morton, P. K., Cambron, S. E., Patrick, K. R., Schemerhorn, B. J. (2010a) Aestivation and diapause syndromes reduce the water balance requirements for pupae of the Hessian fly, *Mayetiola destructor*. *Entomologia Experimentalis et Applicata*, 136, pp. 89–96.

Benoit, J. B., Lopez-Martinez, G., Phillips, Z. P., Patrick, K. R., Denlinger, D. L. (2010b) Heat shock proteins contribute to mosquito dehydration tolerance. *Journal of Insect Physiology,* 56, pp. 151–156.

Block, W., Erzincliogl u, Y. Z., Worland, M. R. (1988) Survival of freezing in *Calliphora* larvae. *CryoLetters,* 9*,* pp. 86-93.

Block, W., Erzinclioglu, Y. Z., Worland, M. R. (1990) Cold Resistance in All Life Stages of 2 Blowfly Species (Diptera, Calliphoridae). *Medical and Veterinary Entomology,* 4, pp. 213-219.

Block, W. (1996) Cold or drought— the lesser of two evils for terrestrial arthropods? *European Journal of Entomology*, 93, pp. 325–339

Bowler, K. (2005) Acclimation, heat shock and hardening. *Journal of Thermal Biology*, 30, pp. 125–130.

Boehme, P., Amendt, J., Zehner, R. (2011) The use of COI barcodes for molecular identification of forensically important fly species in Germany. *Parasitology Research*, 110, pp. 2325–2332.

Bokhorst, S., Huiskes, A., Convey, P., Sinclair, B.J., Lebouvier, M., Vijver, B., Wall, D.H. (2011) Microclimate impacts of passive warming methods in Antarctica: implications for climate change studies*. Polar Biology*, 34, pp. 1421–1435.

Bourel, B., Martin-Bouyer, L., Hedouin, V, Cailliez, J. C., Derout, D., Gosset, D. (1999) Necrophilous insect succession on rabbit carrion in sand dune habitats in northern France. *Journal of Medical Entomology*, 36, pp. 420-425.

Bowen, I. D., Mullarkey, K., Morgan, S. M. (1996) Programmed cell death during metamorphosis in the blow-fly *Calliphora vomitoria*. *Microscopy Research and Technique*, 34, pp. 202–217.

Bradshaw, W. E., Holzapfel, C. M. (2001) Genetic shift in photoperiodic response correlated with global warming. *Proceedings of the National Academy of Sciences of the United States of America*, 98, pp. 14509–14511.

Breshears, D. D., Cobb, N. S., Rich, P. M., Price, K. P., Allen, C. D., Balice, R. G., Romme, W. H., Kastens, J. H., Floyd, M. L., Belnap, J., Anderson, J. J., Myers, O. B., Meyer, C. W. (2005) Regional vegetation die-off in response to global-change-type drought. *Proceedings of the National Academy of Sciences of the United States of America,* 102, pp. 15144–15148.

Brodeur, J. & McNeil, J. N. (1990) Overwintering microhabitat selection by an endoparasitoid (Hymenoptera: Aphidiidae): Induced phototactic and thigmokinetic responses in dying hosts. *Journal of Insect Behavior,* 3, pp. 751-763.

Boufas, G. D. & Koveos, D. S. (2001) Cold hardiness characteristics in a astrain of the predatory mite *Euseius (Amblyseius) finlandicus* (Acari : Phytoseiidae) form northern Greece. *Annals of the Entomological Society of America*, 94, pp. 82-90.

Brown, V. K. (1973) Overwintering Stages *of Ectobius-Lapponicus* (L) (Dictyoptera: Blattidae). *Journal of Entomology Series a-Physiology & Behaviour,* 48, pp. 11-24.

Brown, C. L., Bale J. S. Walters K. F. A. (2004) Freezing induces a loss of freeze tolerance in an overwintering insect. *Proceedings of the Royal Society B. Biological Sciences,* 271, pp. 1507–1511.

Brundage, A., Bros, S., Honda, J. Y. (2011) Seasonal and habitat abundance and distribution of some forensically important blow flies (Diptera: Calliphoridae) in Central California. *Forensic Science International*, 212, pp. 115–120.

Bundy, J. G., Osborn, D., Weeks, J. M., Lindon, J. C., Nicholson, J. K. (2001) An NMR-based metabonomic approach to the investigation of coelomic fluid biochemistry in earthworms under toxic stress. *FEBS Letters,* 500, pp. 31-35.

Burges H. D. (1960) Studies on the dermestid beetle *Trogoderma granarium* everts. IV. Feeding, growth, and respiration with particular reference to diapause larvae. *Journal of Insect Physiology,* 5, pp. 317-334.

Bustard, K. & Gupta, R. S. (1997) The sequences of heat shock protein 40 (DnaJ) homologs provide evidence for a close evolutionary relationship between the Deinococcus-Thermus group and cyanobacteria. *Journal of Molecular Evolution,* 45, pp. 193-205.

Campbell, E. M., Ball, A., Hoppler, S., Bowman, A. S. (2008) Invertebrate aquaporins: a review. *Journal of Comparative Physiology B,* 178, pp. 935–955.

Canzano, A. A., Krockenberger, A. A., Jones, R. E., Seymour, J. E. (2006) Rates of metabolism in diapausing and reproductively active tropical butterflies, *Euploea core* and *Euploea sylvester* (Lepidoptera: Nymphalidae). *Physiological Entomology*, 31, pp. 184–189.

Carpenter J. F., Crowe J. H. (1988) The mechanisms of cryoprotection of proteins by solutes. *Cryobiology,* 25, pp. 244–255.

Chakrabortee, S., Boschetti, C., Walton, L. J., Sarkar, S., Rubinsztein, D. C., Tunnacliffe, A. (2007) Hydrophilic protein associated with desiccation tolerance exhibits broad protein stabilization function. *Proceedings of the National Academy of Sciences of the United States of America,* 104, pp. 18073–18078.

Chen, C. P., Denlinger, D. L., Lee, R. E. (1991a) Seasonal variation in generation time, diapause and cold hardiness in a central Ohio population of the flesh fly, Sarcophaga bullata. *Ecological Entomology*. 16, pp. 155-162

Chen, C. P., Lee, R. E. & Denlinger, D. L. (1991b) Cold Shock and Heat-Shock - a Comparison of the Protection Generated by Brief Pretreatment at Less Severe Temperatures. *Physiological Entomology,* 16, pp. 19-26.

Chen, Q., Ma, E., Behar, K. L., Xu, T., Haddad, G. G. (2002) Role of trehalose phosphate synthase in anoxia tolerance and development in *Drosophila melanogaster*. *Journal of Biological Chemistry*, 277, pp. 3274–3279.

Chernysh, S. I., Simonenko, N. P., Numata, H. (1995) Sensitive stage for the diapause-averting effect of high-temperature in the Blowfly, *Calliphora vicina* Robineau-Desvoidy (Diptera, Calliphoridae). *Applied Entomology and Zoology,* 30, pp. 498-499.

Chino, H. (1957) Conversion of glycogen to sorbitol and glycerol in the diapause egg of the *Bombyx* silkworm. *Nature,* 180, pp. 606-607.

Chown, S. L. & Davis, A. L. V. (2003) Discontinuous gas exchange and the significance of respiratory water loss in scarabaeine beetles. *Journal of Experimental Biology*. 206, pp. 3547–3556.

Chown, S.L. & Nicolson, S.W. (2004) *Insect Physiological Ecology: Mechanisms and Patterns*. Oxford University Press, Oxford.

Chown, S. L., Sorensen, J. G., Terblanche, J. S. (2011) Water loss in insects: an environmental change perspective. *Journal of Insect Physiology*, 57, pp. 1070–1084.

Clough, M. S., Bale, J. S., Harrington, R. (1990) Differential cold hardiness in adults and nymphs of the peach–potato aphid *Myzus persicae*. *Annals of Applied Biology*. 116, pp. 1–9.

Churchill, T. A. & Storey, K. B. (1989) Metabolic Consequences of Rapid Cycles of Temperature-Change for Freeze-Avoiding vs Freeze-Tolerant Insects. *Journal of Insect Physiology,* 35, pp. 579-585.

Cloyd, R. A., Dickinson, A., Larson, R. A., Marley, K. A. (2007). Phototaxis of Fungus Gnat , *Bradysia coprophila* (Lintner) (Diptera: Sciaridae), Adults to Different Light Intensities. *HortScience*, 42, 1217–1220.

Cohen, D. (1966) Optimizing reproduction in a randomly varying environment. *Journal of Theoretical Biology*, 12, pp. 119-129.

Colinet, H., Renault, D., Charoy-Guevel, B., Com, E. (2012a) Metabolic and proteomic profiling of diapause in the aphid parasitoid *Praon volucre*. *PloS One*, 7, pp. e32606.

Colinet, H., Larvor, V., Laparie, M., Renault, D. (2012b). Exploring the plastic response to cold acclimation through metabolomics. *Functional Ecology*, 26, 711–722.

Convey P, Key, R. S., Key R. J. D. (2010) The establishment of a new ecological guild of pollinating insects on sub-Antarctic South Georgia. *Antarctic Science*. 22. pp. 508–512.

Cooper, C. L., Jackowski, S., Rock. C. O. (1987) Fatty acid metabolism in sn-glycerol-3-phosphate acyltransferase (plsB) mutants. *Journal of Bacteriol*ogy. 169, pp. 605–611.

Cornette, R. & Kikawada, T. (2011) The induction of anhydrobiosis in the sleeping chironomid: current status of our knowledge. *IUBMB life*, 63, pp. 419–429.

Costanzo, J. P. & Lee, R. E. (2005). Cryoprotection by urea in a terrestrially hibernating frog. *The Journal of Experimental Biology*, 208, pp. 4079–4089.

Coulson, S. J. & Bale, J. S. (1991) Anoxia induces rapid cold hardening in the housefly *Musca domestica*. *Journal of Insect Physiology.* 37, pp. 497–501.

Crowe, J. H. & Crowe, L. M. (1986) *Stabilisation of membranes in anhydrobiotic organisms.* Leopold, London: Comstock Publishing Association.

Crowe, J. H., Hoekstra, F. A., Crowe, L. M. (1992) Anhydrobiosis. *Annual Review of Physiology,* 54, pp. 579-599.

Crowe, J. H., Carpenter, J. F., Crowe, L. M. (1998). The role of vitrification in anhydrobiosis. *Annual Review of Entomology,* 6, pp. 173-103.

Currah, L. & Ockendon, D. J. (1984) Pollination activity by blowflies and honeybees on onions in breeders' cages. *Annals of Applied Biology*, 105, pp. 167–176.

Dalin, P. (2011) Diapause induction and termination in a commonly univoltine leaf beetle (*Phratora vulgatissima*). *Insect Science*, 18, pp. 443–450.

Danforth, B. N. (1999) Emergence dynamics and bet hedging in a desert bee, *Perdita portalis*. *Proceedings of the Royal Society of London Series B-Biological Sciences,* 266, pp. 1985-1994

Danks, H. (2000) Dehydration in dormant insects. *Journal of Insect Physiology*, 46, pp. 837–852.

Davies, L. (1950). The hatching mechanism of *Muscid* eggs (Diptera). *The Journal of Experimental Biology*, *27*, pp. 437-445.

Davies, L. (1990) Species composition and larval habitats of blow- fly (*Calliphoridae*) populations in upland areas in England and Wales UK*. Medical and Veterinary Entomology,* 4, 61–68.

Davies, L. & Ratcliffe, G. G. (1994) Development Rates of Some Pre-Adult Stages in Blowflies with Reference to Low-Temperatures. *Medical and Veterinary Entomology*, 8, pp. 245-254.

Davies, L. (1999) Seasonal and spatial changes in blowfly production from small and large carcasses at Durham in lowland northeast England. *Medical and Veterinary Entomology*, *13*, pp. 245-251.

Davies, L. (2006) Lifetime reproductive output of *Calliphora vicina* and *Lucilia sericata* in outdoor caged and field populations; flight vs. egg production? *Medical and Veterinary Entomology,* 20, pp. 453-458.

Dedeine, F., Vavre, F., Fleury, F., Loppin, B., Hochberg, M. E., Bouletreau, M. (2001) Removing symbiotic *Wolbachia* bacteria specifically inhibits oogenesis in a parasitic wasp. *Proceedings of the National Academy of Sciences of the United States of America*, 98, pp. 6247–6252.

de la Roche, M., Tessier, S. N., Storey, K. B. (2012). Structural and functional properties of glycerol-3-phosphate dehydrogenase from a mammalian hibernator. *The Protein Journal*, 31, 109–119.

Denlinger, D. L. (1971) Embryonic determination of pupal diapause in the flesh fly *Sarcophaga crassipalpis*. *Journal of Insect Physiology*, 17, pp. 1815-1822.

Denlinger, D. L., (1972) Seasonal phenology of diapause in the flesh fly *Sarcophaga bullata*. *Annals of the Entomological Society of America,* 65, pp. 410–414.

Denlinger, D. L. (1979) Pupal diapause in tropical flesh flies: environmental and endocrine regulation, metabolic rate and genetic selection. In: Izumi, Y., Sonoda, S., Tsumuki, H. (2007) Effects of diapause and cold-acclimation on the avoidance of freezing injury in fat body tissue of the rice stem borer, *Chilo suppressalis* Walker. *Journal of Insect Physiology*, 53, pp. 685-690.

Denlinger, D. L. (1985) Hormonal control of diapause. *In: Comprehensive Insect Physiology, Biochemistry and Pharmacology, ed.* G. A. Kerkut, L. I. Gilbert. Oxford: Pergamon, pp. 353–412.

Denlinger, D. L. (1986) Dormancy in Tropical Insects*. Annual Review of Entomology*, 31, pp. 239-264.

Denlinger, D. L. & Bradfield, J. Y. (1980) Programming the Duration of Pupal Diapause in the Tobacco Hornworm. *American Zoologist,* 20, pp. 937-937.

Denlinger, D. L. (1991) Relationship between cold hardiness and diapause. In: *Insects at Low Temperature*. Lee, R. E. & Denlinger, D. L. (Eds.), Chapman and Hall, New York and London. pp. 174–198.

Denlinger, D. L. (2002) Regulation of diapause. *Annual Review of Entomology,* 47, pp. 93-122.

Denlinger, D .L. & Lee, R. E. (2010) *Low Temperature Biology of Insects*. Cambridge University Press, Cambridge.

Devries, A. L. (1971) Glycoproteins as Biological Antifreeze Agents in Antarctic Fishes. *Science,* 172, pp. 1152-1155.

Diamant, S., Eliahu, N., Rosenthal, D., Goloubinoff, P. (2001) Chemical chaperones regulate molecular chaperones in vitro and in cells under combined salt and heat stresses. *Journal of Biological Chemistry.* 276, pp. 39586-39591.

Dillon, M. E., Wang, G., Huey, R. B., (2010) Global metabolic impacts of recent climate warming. *Nature*, 467, pp. 704–706.

Dockx, C. (2012) Differences in phenotypic traits and migratory strategies between eastern North American monarch butterflies, *Danaus plexippus* (L.). *Biological Journal of the Linnean Society*, 106, pp. 717–736.

Dolezal, P. & Sehnal, F. (2007) Effects of photoperiod and temperature on the development and diapause of the bark beetle *Ips typographus*. *Journal of Applied Entomology,* 131, pp. 165-173.

Donovan, S. E., Hall, M. J. R., Turner, B. D., Moncrieff, C. B. (2006) Larval growth rates of the blowfly, *Calliphora vicina*, over a range of temperatures. *Medical and Veterinary Entomology*, 20, pp. 106–114.

Dowhan, W. (1997) Molecular basis for membrane phospholipid diversity: Why are there so many lipids? *Annual Reviews of Biochemistry.* 66, pp. 189-194.

Drobnis, E. Z., Crowe, L. M., Berger, T., Anchordoguy, T. J., Overstreet, J. W., Crowe, J. H. (1993) Cold Shock Damage Is Due to Lipid Phase-Transitions in Cell-Membranes - a Demonstration Using Sperm as a Model. *Journal of Experimental Zoology,* 265, pp. 432-437.

Duman, J. G. & DeVries, A. L. (1972) Freezing Behavior of Aqueous-Solutions of Glycoproteins from Blood of an Antarctic Fish. *Cryobiology,* 9, pp. 469-472.

Duman J. G., & DeVries, A. L. (1974) Effects of temperature and photoperiod on antifreeze production in cold water fishes. *Journal of Experimental Biology*, 190, pp. 89-97.

Duman, J. G., Wu, D. W., Xu, L., Tursman, D. Olsen, T. M. (1991). Adaptations of insects to subzero temperatures. *Quarterly Review of Biology,* 66, pp. 387-410.

Duman, J. G. & Olsen, T. M. (1993) Thermal Hysteresis Protein Activity in Bacteria, Fungi, and Phylogenetically Diverse Plants. *Cryobiology*, 30, pp. 322-328.

Duman, J. G., Li, N., Verleye, D., Goetz, F. W., Wu, D. W., Andorfer, C. A., Benjamin, T., Parmelee, D. C. (1998) Molecular characterization and sequencing of antifreeze proteins from larvae of the beetle *Dendroides canadensis*. *Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology,* 168, pp. 225-232.

Duman, J. G., Bennett, V., Sformo, T., Hochstrasser, R., Barnes, B. M. (2004) Antifreeze proteins in Alaskan insects and spiders. *Journal of Insect Physiology,* 50, pp. 259-266.

Durner, G. M., Whiteman, J. P., Harlow, H. J., Amstrup, S. C., Regehr, E. V., Ben-David, M. (2011) Consequences of long-distance swimming and travel over deep-water pack ice for a female polar bear during a year of extreme sea ice retreat. *Polar Biology*, 34, pp. 975–984.

Easterling, D. R., Meehl, G. A., Parmesan, C., Changnon, S. A., Karl, T. R., Mearns, L. O. (2000) Climate extremes: observations, modeling, and impacts. *Science*, 289, pp. 2068–2074.

Edney, E. B. (1971). Some aspects of water balance in tenebrionid beetles and a thysanuran from the Namib Desert of southern Africa. *Physiological. Zoology,* 44, pp. 61–76.

Edwards, J. P., Corbitt, T. S., McArdle, H. F., Short, J. E., Weaver, R. J. (1995) Endogenous levels of insect juvenile hormones in larval, pupal and adult stages of the tomato moth, *Lacanobia oleracea. Journal of Insect Physiology*, 41, pp. 641–651.

Eizaguirre, M., Lopez, C., Albajes, R. (2008) Factors affecting the natural duration of diapause and post-diapause development in the Mediterranean corn borer *Sesamia nonagrioides* (Lepidoptera: Noctuidae). *Journal of Insect Physiology*, 54, pp. 1057–1063.

Ellis, J. K., Athersuch, T. J., Cavill, R., Radford, R., Slattery, C., Jennings, P., McMorrow, T., Ryan, M. P., Ebbels, T. M. D., Keun, H. C. (2011) Metabolic response to low-level toxicant exposure in a novel renal tubule epithelial cell system. *Molecular Biosystems,* 7, pp. 247-257.

Emerson, K. J., Bradshaw, W. E., Holzapfel, C. M. (2010) Microarrays reveal early transcriptional events during the termination of larval diapause in natural populations of the mosquito, *Wyeomyia smithii*. *PloS One*. 5, pp. e9574.

Erwin, T. L. (1982) Tropical forests: their richness in *Coleoptera* and other arthropod species. *Coleopterists Bulletin* 36, pp. 74–75.

Erzinclioglu, Y. Z. (1996). *Blowflies, Naturalists' Handbook* 23. 1st Edition. Richmond.

Evans, D. L. (2000) *Insect Defenses: adaptive mechanisms and strategies of prey and predators*. State University Press: New York, N.Y., USA.

Fantinou, A. A., Kourti, A. T., Saitanis, C. J. (2003) Photoperiodic and temperature effects on the intensity of larval diapause in *Sesamia nonagrioides*. *Physiological Entomology,* 28, pp. 82-87.

Feder, M. E., & Hofmann, G. E. (1999) Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annual Review of Physiology*, 61, pp. 243–282.

Feidantsis, K., Anestis, A., Vasara, E., Kyriakopoulou-Sklavounou, P., Michaelidis, B. (2012) Seasonal variations of cellular stress response in the heart and gastrocnemius muscle of the water

frog (*Pelophylax ridibundus*). *Comparative Biochemistry and Physiology. Part A, Molecular & integrative physiology, 162,* pp. 331–339.

Fields, P. G., Fleurat-Lessard, F., Lavenseau, L., Febvay, G., Peypelut, L. & Bonnot, G. (1998) The effect of cold acclimation and deacclimation on cold tolerance, trehalose and free amino acid levels in *Sitophilus granarius* and *Cryptolestes ferrugineus* (Coleoptera). *Journal of Insect Physiology*, 44, pp. 955-965.

Fink, A. L. (1999) Chaperone-mediated protein folding. *Physiological Reviews,* 79, pp. 425-449.

Fleming, J. E., Walton, J. K., Dubitsky, R., Bensch, K. G. (1988) Aging results in an unusual expression of *Drosophila* heat shock proteins. *Proceedings of the National Academy of Sciences of the United States of America*, 85, pp. 4099–4103.

Forrest, J., & Miller-Rushing, A.J. (2010) Toward a synthetic understanding of the role of phenology in ecology and evolution. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, 365, pp. 3101–12.

Finney, D. J. (1971) *Probit analysis*. University Press, Cambridge.

Fischer, O. A., Matlova, L., Dvorska, L., Svastova, P., Bartl, J., Weston, R. T., Pavlik, I. (2004) Blowflies *Calliphora vicina* and *Lucilia sericata* as passive vectors of *Mycobacterium avium* subsp. *avium*, *M. a. paratuberculosis* and *M. a. hominissuis*. *Medical and veterinary entomology*, 18, pp. 116–122.

Fraenkel, G. & Bhaskaran. G. (1973) Pupariation and pupation in *Cyclorrhaphous* flies (Diptera): Terminology and interpretation. *Annals of the Entomological Society of America,* 66, pp. 418-422.

Franck, E., Madsen, O., Van Rheede, T., Ricard, G., Huynen, M. A., De Jong, W. W. (2004) Evolutionary diversity of vertebrate small heat shock proteins. *Journal of Molecular Evolution,* 59, pp. 792-805.

Franek, F. & Sramkova, K. (1996) Protection of B lymphocyte hybridoma against starvation-induced apoptosis: Survival-signal role of some amino acids. *Immunology Letters,* 52, pp. 139-144.

Fraser, A. (1959) Neurosecretion in the brain of the larva of the sheep blowfly, *Lucilia caesar*. *Quarterly Journal of Microscopial Science,* 100, pp. 377-399.

Fry, J. C. (1993) *Biological data analysis: a practical approach*. University Press, Oxford.

Fuchs, B. C., Bode, B. P. (2006) Stressing out over survival: glutamine as an apoptotic modulator. *The Journal of Surgical Research*, 131, pp. 26–40.

Gadenne, C., Dufour, M. C., Rossignol, F., Becard, J. M., Couillaud, F. (1997) Occurrence of non-stationary larval moults during diapause in the corn-stalk borer, *Sesamia nonagrioides* (Lepidoptera: Noctuidae). *Journal of Insect Physiology*, 43, pp. 425–431.

Gallai, N., Salles, J. M., Settele, J., Vaissiere, B. E. (2009) Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. *Ecological Economics*, 68, pp. 810–821.

Gennard, D. E. (2007) *Forensic Entomology: An Introduction*, First ed., John Wiley & Sons Inc., West Sussex.

Gibbs, A. G., Chippindale, A. K., Rose, M. R. (1997) Physiological mechanisms of evolved desiccation resistance in *Drosophila melanogaster*. *The Journal of Experimental Biology*, 200, pp. 1821–1832.

Godfree R, Roberston B, Bolger T, Carnegie M, Young A (2011) An improved hexagon open-top chamber system for stable diurnal and nocturnal warming and atmospheric carbon dioxide enrichment. *Global Change Biology*, 17, pp.439–451

Godfrey, H. C. J. & Hassell, M. P. (1987) Natural enemies may be a cause of discrete generations in tropical insects. *Nature,* 327, pp. 144–147.

Gomi, T., Nagasaka, M., Fukuda, T., Hagihara, H. (2007) Shifting of the life cycle and life-history traits of the fall webworm in relation to climate change. *Entomologia Experimentalis et Applicata*, 125, pp. 179–184.

Gordo, O., Sanz, J. J. (2005) Phenology and climate change: a long-term study in a Mediterranean locality. *Oecologia*, 146, pp. 484–95.

Goto, S. G., Yoshida, K. M., Kimura, M. T. (1998a) Accumulation of Hsp70 mRNA under environmental stresses in diapausing and nondiapausing adults of *Drosophila triauraria*. *Journal of Insect Physiology,* 44, pp. 1009–1015.

Goto, M., Fujii, M., Suzuk,i K., Sakai, M. (1998b) Factors affecting carbohydrate and free amino acid content in overwintering larvae of *Enosima leucotaeniella*. *Journal of Insect Physiology* ,44, pp. 87–94.

Goto, M., Sekine, Y., Outa, H., Hujikura, M., Koichi, S. (2001) Relationships between cold hardiness and diapause, and between glycerol and free amino acid contents in overwintering larvae of the oriental corn borer, *Ostrinia furnacalis*. *Journal of Insect Physiology*, 47, pp. 157–165.

Goto, S.G. & Katagiri, C. (2011) Effects of acclimation temperature on membrane phospholipids in the flesh fly *Sarcophaga similis*. *Entomological Science*, 14, pp. 224–229.

Graham, L. A., Liou, Y. C., Walker, V. K., Davies, P. L. (1997) Hyperactive antifreeze protein from beetles. *Nature,* 388, pp. 727-728.

Graham, L. A. & Davies, P. L. (2005) Glycine-rich antifreeze proteins from snow fleas. *Science,* 310, pp. 461-461.

Graham-Smith, G. S. (1916) Observations on the habits and parasites of common flies. *Parasitology*, 8, pp. 440–544.

Grassberger, M. & Reiter, C. (2002) Effect of temperature on development of the forensically important holarctic blow fly *Protophormia terraenovae* (Robineau-Desvoidy) (Diptera: Calliphoridae). *Forensic Science International,* 128, pp. 177-182.

Greenberg, B. (1973) *Flies and Disease. Biology and Disease Transmission.* Princeton University Press: Princeton, NJ, USA.

Greenberg, B. (1991) Flies as forensic indicators. *The Journal of Medical Entomology*. 28, pp. 565–577.

Greenberg, B. & Kunich J. C. (2002) *Entomology and the law: flies as forensic indicators*. Cambridge University Press, Cambridge.

Gullan, P. J. & Cranston, P. S. (2005) *The Insects: an outline of entomology.* Blackwell Publishing LTD, Bath.

Gunn, A., & Bird, J. (2011) The ability of the blowflies *Calliphora vomitoria* (Linnaeus), *Calliphora vicina* (Rob-Desvoidy) and *Lucilia sericata* (Meigen) (Diptera: Calliphoridae) and the muscid flies *Muscina stabulans* (Fallén) and *Muscina prolapsa* (Harris) (Diptera: Muscidae) to colonise buried remains. *Forensic Science International*, 207, pp. 198–204.

Gusev, O., Cornette, R., Kikawada, T., Okuda, T. (2011) Expression of heat shock protein-coding genes associated with anhydrobiosis in an African chironomid *Polypedilum vanderplanki*. *Cell stress & chaperones*, 16, pp. 81–90.

Hadley, N. F. (1994) *Water relations of terrestrial arthropods*. Academic Press, New York.

Hahn, D. A. & Denlinger, D. L. (2007) Meeting the energetic demands of insect diapause: nutrient storage and utilization. *Journal of Insect Physiology*. 53, pp. 760–773.

Haines, T. H. (2001) Do sterols reduce proton and sodium leaks through lipid bilayers? *Progress in Lipid Research*, 40, pp. 299–324.

Han, R., Ge, F., Yardim, E. N., He, Z. (2005) The effect of low temperatures on diapause and non-diapause larvae of the pine caterpillar, *Dendrolimus tabulaeformis* Tsai et Liu (Lepidoptera: Lasiocampidae). *Applied Entomology and Zoology*, 40, pp. 429–435.

Hand, S. C., Menze, M. A., Toner, M., Boswell, L., Moore, D. (2011) LEA proteins during water stress: not just for plants anymore. *Annual Review of Physiology*, 73, pp. 115–134.

Hanski, I. (1988) 4 kinds of extra long diapause in insects - a review of theory and observations. *Annales Zoologici Fennici,* 25, pp. 37-53.

Hanski, I. (1989) Prolonged diapause: theory and observations. In proceeding: *Cone and seed insects working party conference. Working party S2.07-01, IUFRO, 26-30 June 1988* (ed G.E. Miller), pp. 91-100. Victoria, BC, Forestry Canada, Pacific Forestry Centre, Victoria, BC, Canada.

Hatherly, I. S., Bale, J. S., Walters, K. F. A., Worland, M. R. (2004) Thermal biology of *Typhlodromips montdorensis*: implications for its introduction as a glasshouse biological control agent in the UK. *Entomologia Experimentalis et Applicata*, 111, pp. 97–109.

Hart, A. J. & Bale, J. S. (1997) Evidence for the first strongly freeze-tolerant insect found in the U.K. *Ecological Entomology*, 22, pp. 242–245.

Hart, A. J. & Bale, J. S. (1998) Factors Affecting the Freeze Tolerance of the Hoverfly *Syrphus ribesii* (Diptera: Syrphidae). *Journal of Insect Physiology*, 44, pp. 21–29.

Hart, A. J., Bale, J. S., Tullett, A. G., Worland, M. R., Walters, K. F. A. (2002) Effects of temperature on the establishment potential of the predatory mite *Amblyseius californicus* McGregor (Acari: Phytoseiidae) in the UK. *Journal of Insect Physiology*, 48, pp. 593–599.

Hartl, F. U., Hayer-Hartl, M. (2002) Molecular chaperones in the cytosol: from nascent chain to folded protein. *Science*, 295, pp. 1852–1858.

Harvey, M. L., Gaudieri, S., Villet, M. H., Dadour, I. R. (2008) A global study of forensically significant calliphorids: implications for identification. *Forensic science international*, 177, pp. 66–76.

Haskell, N. H., Lord, W.D., Byrd, J. F. (2000) Collection of entomological evidence during death investigations. In: *Forensic Entomology: the Utility of Arthropods in Legal Investigations* (ed. by J. H. Byrd and J. L. Castner), CRC Press, Florida. pp. 81–120.

Hatherly, I. S., Pedersen, B. P., Bale, J. S. (2008) Establishment potential of the predatory mirid *Dicyphus hesperus* in northern Europe. *BioControl*, 53, pp. 589–601.

Hawes, T. C., Hines, A. X., Viant, M. R., Bale. J. S. (2008) The metabolomic fingerprint of cryo-stress in a freeze tolerant insect. *CryoLetters*, 29, pp. 505–515.

Hawes, T. C. & Wharton, D. A. (2010) Tolerance of freezing in caterpillars of the New Zealand Magpie moth (*Nyctemera annulata*). *Physiological Entomology*, 35, pp. 296–300.

Hawes, T. C., Marshall, C. J., Wharton, D. A. (2011) Antifreeze proteins in the Antarctic springtail, *Gressittacantha terranova*. *Journal of Comparative Physiology. B, Biochemical, Systemic, and Environmental Physiology*, 181, pp. 713–719.

Hayward, S. A. L., Rinehart, J. P., Denlinger, D. L. (2004) Desiccation and rehydration elicit distinct heat shock protein transcript responses in flesh fly pupae. *Journal of Experimental Biology.* 207, pp. 963-971.

Hayward, S. A. L., Pavlides, S. C., Tammariello, S. P., Rinehart, J. P., Denlinger, D. L. (2005) Temporal expression patterns of diapause-associated genes in flesh fly pupae from the onset of diapause through post-diapause quiescence. *Journal of Insect Physiology,* 51, pp. 631–640.

Hayward, S. A. L., Rinehart, J. P., Sandro, L. H., Lee, R. E., Denlinger, D. L. (2007) Slow dehydration promotes desiccation and freeze tolerance in the Antarctic midge *Belgica antarctica*. *The Journal of Experimental Biology*, 210, pp. 836–844.

Hazel, W. N. & West, D. A. (1983) The effect of larval photoperiod on pupal color and diapause in swallowtail butterflies. *Ecological Entomology,* 8, pp. 37-42.

Hazel, J. R. (1989) Cold adaptation in ectotherms: regulation of membrane function and cellular metabolism. *Advances in Comparative and Environmental Physiology*, 4, pp. 1- 50.

Hazel, J. R. (1995) Thermal adaptation in biological-membranes - is homeoviscous adaptation the explanation. *Annual Review of Physiology*, 57, pp. 19-42.

He, H. M., Xian, Z. H., Huang, F., Liu, X. P., Xue, F. S. (2009) Photoperiodism of diapause induction in *Thyrassia penangae* (Lepidoptera: Zygaenidae). *Journal of Insect Physiology*, 55, pp. 1003–1008.

Henning, W. (1950) Entomologishe Beobachtungen an kleinen Wirbeltierleichen. *Zeitschrift fur Zoology,* 38, 33-88. In: *Forensic Entomology: The Utility of Arthropods in Legal Investigations.* Byrd, J. H. & Castner, J. L.(Eds.) CRC Press, Florida.

Herman, W. S. (1981) Studies on the adult reproductive diapause of the monarch butterflies, *Danaus plexippus*. *Biological Bulletin*. 160, pp. 89-106.

Hetz, S. K. & Bradley, T. J. (2005) Insects breathe discontinuously to avoid oxygen toxicity. *Nature,* 433, pp. 516-519.

Higley, L.G. & Haskell, N.H. (2001) *Insect development and forensic entomology.*(ed. by J. H. Byrd and J. L. Castner), CRC Press. pp. 287–302.

Hilgenboecker, K., Hammerstein, P., Schlattmann, P., Telschow, A., Werren, J. H. (2008) How many species are infected with *Wolbachia*?--A statistical analysis of current data. *FEMS Microbiology Letters*, 281, pp. 215–220.

Hodek, I. (1983) Role of environmental factors and endogenous mechanisms in the seasonality of reproduction in insects diapausing as adults. *In: Brown, V.K., Hodek, I. (Eds.), Diapause and Life Cycle Strategies in Insects*. Dr W Junk Publishers, The Hague. pp. 9–33.

Hodek, I. (1996) Diapause development, diapause termination and the end of diapause. *European Journal of Entomology*, 93, pp. 475–487.

Hodkova, M. & Hodek, I. (1994) Control of diapause and supercooling by the retrocerebral complex in *Pyrrhocoris apterus*. *Entomologia Experimentalis et Applicata,* 70, pp. 237–245.

Hodkova, M. & Hodek, I. (1997) Temperature regulation of supercooling and gut nucleation in relation to diapause of Pyrrhocoris apterus (L.) (Heteroptera). *Cryobiology,* 34, pp. 70–79.

Hodkova, M. & Hodek, I. (2004) Photoperiod, diapause and cold-hardiness. *European Journal of Entomology,* 101, pp. 445–458.

Holmstrup, M., & Westh, P. (1994) Dehydration of earthworm cocoons exposed to cold: a novel cold hardiness mechanism. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology*, 164, pp. 312– 315.

Holmstrup, M., & Somme, L. (1998) Dehydration and cold hardiness in the Arctic Collembolan *Onychiurus arcticus Tullberg* 1876. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology*, 168 , pp. 197–203.

Holmstrup, M., Costanzo, J. P., Lee, R. E. (1999) Cryoprotective and osmotic responses to cold acclimation and freezing in freeze-tolerant and freeze-intolerant earthworms. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology*, 169, pp. 207–214.

Holmstrup, M. (2002) *Strategies for cold and drought tolerance in permeable soil invertebrates*. Doctor's dissertations (Dsc). National Environmental Research Institute, Silkeborg, Denmark.

Holmstrup, M., Bayley, M., Pedersen, S. A., Zachariassen, K. E. (2010*) Interaction between cold, desiccation and environmental toxins*. In: Low Temperature Biology of Insects. Denlinger, D.L. & Lee, R.E. (Eds.), Cambridge University Press, Cambridge, pp. 166–187.

Hou, M., Han, Y., Lin, W. (2009) Influence of soil moisture on supercooling capacity and associated physiological parameters of overwintering larvae of rice stem borer. *Entomological Science*, 12, pp. 155–161.

Howlett, B. G. (2011) Hybrid carrot seed crop pollination by the fly *Calliphora vicina* (Diptera: Calliphoridae). *Journal of Applied Entomology*, 136, pp. 421–430.

Howling, G. G., Bale, J. S., Harrington, R. (1994) Effects of extended and repeated exposures on the low temperature mortality of the peach–potato aphid *Myzus persicae*. *Ecological Entomology*, 19, pp. 361–366.

Huang, C., Lin, H., Li, S., Wang, G. (1997) Influence of the positions of cis double bonds in the sn-2-acyl chain of phosphatidylethanolamine on the bilayer's melting behavior. *The Journal of Biological Chemistry*, 272, pp. 21917–21926.

Hughes, G. E., Bale, J. S., Sterk, G. (2009) Thermal biology and establishment potential in temperate climates of the predatory mirid *Nesidiocoris tenuis*. *BioControl*, 54, pp. 785–795.

Hughes, G. E., Sterk, G., Bale, J. S. (2011) Thermal biology and establishment potential in temperate climates of the aphid parasitoid, *Lysiphlebus testaceipes*. *BioControl*, 56, pp. 19–33.

Hull-Sanders, H. M., Appel, A. G., Eubanks, M. D. (2003) Comparative water relations of adult and juvenile tortoise beetles: differences among sympatric species. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 135, pp. 625–634.

Hulthen, A. D. & Clarke, A. R. (2006) The influence of soil type and moisture on pupal survival of *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae). *Australian Journal of Entomology*, 45, pp. 16–19.

Hurcus, M. J. & Hoffmann, A. A. (1999) Desiccation resistance in interspecific *Drosophila* crosses: genetic interactions and Trait correlations. *Genetics*, 151, pp. 1490-1502.

Hwang, C. & Turner, B. D. (2005) Spatial and temporal variability of necrophagous *Diptera* from urban to rural areas. *Medical and Veterinary Entomology,* 19, pp. 379-391.

IPCC, (2007) *Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change*. Solomon, S., D. Qin, M. Manning, Z. Chen, M. Marquis, K.B. Averyt, M. Tignor and H.L. Miller (eds.) Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.

Irwin, J. T. & Lee, R. E. (2002). Energy and water conservation in frozen vs. supercooled larvae of the goldenrod gall fly, *Eurosta solidaginis* (Fitch) (Diptera: Tephritidae). *Journal of Experimental Zoology*, 292, pp. 345-350.

Irwin, J. T. & Lee, R. E. (2003) Cold winter microenvironments conserve energy and improve overwintering survival and potential fecundity of the goldenrod gall fly, *Eurosta solidaginis. OIKOS*, 1, pp. 71–78.

Ishiguro, S., Li, Y., Nakano, K., Tsumuki, H., Goto, M. (2007) Seasonal changes in glycerol content and cold hardiness in two ecotypes of the rice stem borer, *Chilo suppressalis*, exposed to the environment in the Shonai district, Japan. *Journal of Insect Physiology*, 53, pp. 392–397.

Ishiwata, A., Sakurai, A., Nishimiya, Y., Tsuda, S., Ito, Y. (2011) Synthetic study and structural analysis of the antifreeze agent xylomannan from *Upis ceramboides*. *Journal of the American Chemical Society*, 133, pp. 19524–19535.

Iwasa, M. & Inoue, A. (2012) Seasonal and spatial distributions in relation to reproduction of blowflies (Diptera, Calliphoridae) in Hokkaido, Japan. *Medical Entomology and Zoology*, 63, pp. 1-10

Iwata, K. I., Fujiwara, Y., Takeda, M. (2005) Effects of temperature, sorbitol, alanine and diapause hormone on the embryonic development in *Bombyx mori*: in vitro tests of old hypotheses. *Physiological Entomology*, 30, pp. 317–323.

Izumi, Y., Katagiri, C., Sonoda, S., Tsumuki, H. (2009) Seasonal changes of phospholipids in last instar larvae of rice stem borer *Chilo suppressalis* Walker (Lepidoptera: Pyralidae). *Entomological Science*, 12, pp. 376–381.

Jensen, D., Overgaard, J., Sorensen, J. G. (2007). The influence of developmental stage on cold shock resistance and ability to cold- harden in *Drosophila melanogaster. Journal of Insect Physiology,* 53, pp. 179–186.

Jing, X. H., Kang, L. (2003) Geographical variation in egg cold hardiness: a study on the adaptation strategies of the migratory locust Locusta migratoria L. *Ecological Entomology*, 28, pp. 151–158.

Joblin, K. H., Yocum, G. D., Denlinger, D. L. (1990) Cold shock elicits expression of heat shock proteins in the flesh fly, *Sarcophaga crassipalpis. Journal of Insect Physiology*, 36, pp. 825-834.

Johnsen, S., Kelber, A., Warrant, E., Sweeney, A. M., Widder, E. A, Lee, R. L., Hernandez-Andres, J. (2006) Crepuscular and nocturnal illumination and its effects on color perception by the nocturnal hawkmoth *Deilephila elpenor*. *The Journal of Experimental Biology*, 209, pp. 789–800.

Kageyama, T. & Ohnishi, E. (1971) Carbohydrate Metabolism in Eggs of Silkworm, *Bombyx-Mori*. Absence of Phosphofructokinase in Diapausing Egg. *Development Growth & Differentiation*, 13, pp. 97-104.

Kaneshrajah G. & Turner B. (2004) *Calliphora vicina* larvae grow at different rates on different body tissues*. International Journal of Legal Medicine*. 118, pp. 242-244

Kaneko, J., Kita, K., Tanno, K. (1991) INA bacteria isolated from diamondback moth, *Plutella xylostella* L. pupae (Lepidoptera: Yponomeutidae). *Japanese Journal of Applied Entomology and Zoology,* 35, pp. 7-11.

Kang, S. M., Park, J. C., Shin, M. J., Lee, H., Oh, J., Ryu, D. H., Hwang, G. S. Chung, J. H. (2011) H-1 nuclear magnetic resonance based metabolic urinary profiling of patients with ischemic heart failure. *Clinical Biochemistry,* 44, pp. 293-299.

Kawano, T., Shimoda, M., Matsumoto, H., Ryuda, M., Tsuzuki, S., Hayakawa, Y. (2010) Identification of a gene, Desiccate, contributing to desiccation resistance in *Drosophila melanogaster*. *The Journal of Biological Chemistry*, 285, pp. 38889–38897.

Kellermann, V., Overgaard, J., Hoffmann, A. A., Flojgaard, C., Svenning, J. C., Loeschcke, V. (2012) Upper thermal limits of Drosophila are linked to species distributions and strongly constrained phylogenetically. *Proceedings of the National Academy of Sciences of the United States of America*, 109, pp. 16228–16233.

Kelty, J. D., Lee, R. E. (1999) Induction of rapid cold hardening by cooling at ecologically relevant rates in *Drosophila melanogaster*. *Journal of Insect Physiology*, 45, pp. 719–726.

Khani, A., Moharramipour, S., Barzegar, M. (2007) Cold tolerance and trehalose accumulation in overwintering larvae of the codling moth, *Cydia pomonella* (Lepidoptera : Tortricidae). *European Journal of Entomology*, 104, pp. 385-392.

Khani, A., Moharramipour, S. (2010) Cold hardiness and supercooling capacity in the overwintering larvae of the codling moth, *Cydia pomonella*. *Journal of Insect Science*, 10, p. 83.

Khodayari, S., Moharramipour, S., Kamali, K., Jalali Javaran, M., Renault, D. (2012) Effects of acclimation and diapause on the thermal tolerance of the two-spotted spider mite *Tetranychus urticae*. *Journal of Thermal Biology*, 37, pp. 419–423.

Kikawada, T., Nakahara, Y., Kanamori, Y., Iwata, K. I., Watanabe, M., McGee, B., Tunnacliffe, A., Okuda, T. (2006) Dehydration-induced expression of LEA proteins in an anhydrobiotic chironomid. *Biochemical and Biophysical Research Communications*. 348, pp. 56–61.

Kikawada, T., Saito, A., Kanamori, Y., Nakahara, Y., Iwata, K. I., Tanaka, D., Watanabe, M., Okuda, T. (2007) Trehalose transporter 1, a facilitated and high-capacity trehalose transporter, allows exogenous trehalose uptake into cells*. Proceedings of the National Academy of Sciences of the United States of America*, 104, pp. 11585–11590.

Kim, Y., Kim, N. (1997). Cold hardiness in *Spodoptera exigua* (Lepidoptera: Noctuiae). *Environmental Entomology*, 26, pp. 1117–1123.

Kimura, M. T. & Minami, N. (1980) Reproductive diapause in *Drosophila auraria* sensitivity of different developmental stages to photoperiod and temperature. *Kontyu,* 48, pp. 458-463.

Kingslover, J. & Nagle, A. (2007) Evolutionary divergence in thermal sensitivity and diapause of field and laboratory populations of *Manduca sexta*. *Physiological and Biochemical Zoology*. 80, pp. 473-479.

Kiritani, K. (1971) Distribution and abundance of the southern green stink bug, *Nezara viridula*. *In: Proceedings of the Symposium on Rice Insects*, pp. 235–248. Tropical Agricultural Research Center, Tokyo.

Kiritani, K., Hokyo, N., Kimura, K. (1966) Factors affecting the winter mortality in the southern green stink bug, *Nezara viridula*. *Annales de la Société Entomologique de France, Nouvelle Série*, 2, pp. 199–207.

Klein, M. S., Dorn, C., Saugspier, M., Hellerbrand, C., Oefner, P. J., Gronwald, W. (2011) Discrimination of steatosis and NASH in mice using nuclear magnetic resonance spectroscopy. *Metabolomics.* 7, pp. 237-246.

Knight, C. A. (1967) *The freezing of supercooled liquids*. New York: VanNostrand.

Knight, J.D. (1987) Cold hardening and overwintering survival of the grain aphid *Sitobion avenae* in Northern England, PhD thesis. University of Leeds.

Kluser, S. & Peduzzi P. (2007) *Global pollinator decline: a literature review*. Geneva: UNEP/GRID.

Kono, Y. (1980) Endocrine activities and photoperiodic sensitivity during pre-diapause period in the phytophagous lady beetle, *Epilachna Vigintioctopunctata*. *Applied Entomology and Zoology,* 15, pp. 73-80.

Kostal, V., Haverlka, J. (2000) Diapausing larvae of the midge *Aphidoletes aphidimyza* (Diptera: Cecidomyiidae) survive at subzero temperatures in a supercooled state but tolerate freezing if inoculated by external ice. *European Journal of Entomology*, 97, pp. 433–436.

Kostal, V., Slachta, M., Simek, P. (2001) Cryoprotective role of polyols independent of the increase in supercooling capacity in diapausing adults of *Pyrrhocoris apterus* (Heteroptera: Insecta). *Comparative biochemistry and physiology. Part B, Biochemistry & Molecular Biology*, 130, pp. 365–74.

Kostal, V., Berkova, P., Simek, P. (2003) Remodelling of membrane phospholipids during transition to diapause and cold-acclimation in the larvae of *Chymomyza costata* (Drosophilidae). *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology,* 135, pp. 407-419.

Kostal, V., Tamura, M., Tolarova, M., Zahradnıckova, H. (2004a) Enzymatic capacity for accumulation of polyol cryoprotectants changes during diapause development in the adult red firebug, *Pyrrhocoris apterus*. *Physiological Entomology.* 29, pp. 344–355.

Kostal, V., Tollarova, M., Sula, J. (2004b) Adjustments of the enzymatic complement for polyol biosynthesis and accumulation in diapause cold-acclimated adults of *Pyrrhocoris apterus*. *Journal of Insect Physiology*, 50, pp. 303–313.

Kostal, V. (2006) Eco-physiological phases of insect diapause. *Journal of Insect Physiology,* 52, pp. 113-127.

Kostal, V. (2010) Cell structural modifications in insects at low temperatures. In: *Low Temperature Biology of Insects,* Denlinger, D. L. & Lee, R. E. (Eds.), Cambridge University Press, Cambridge, pp. 116-140.

Kostal, V., Dolezal, P., Rozsypal, J., Moravcova, M., Zahradníckova, H., Simek, P. (2011a) Physiological and biochemical analysis of overwintering and cold tolerance in two Central European populations of the spruce bark beetle, *Ips typographus*. *Journal of Insect Physiology*, 57, pp. 1136–1146.

Kostal, V., Zahradníckova, H., Simek, P. (2011b) From the Cover: Hyperprolinemic larvae of the drosophilid fly, *Chymomyza costata*, survive cryopreservation in liquid nitrogen. *Proceedings of the National Academy of Sciences of the United States of America*, 108, pp. 13041–13046.

Kostal, V., Simek, P., Zahradnickova, H., Cimlova, J., Stetina, T. (2012) Conversion of the chill susceptible fruit fly larva (*Drosophila melanogaster*) to a freeze tolerant organism. *PNAS*, 109, pp. 3270–3274.

Koveos, D. S. & Broufas, G. D. (2001) Survival under dry conditions of males, and diapause and non-diapause females of the predatory mite *Euseius finlandicus* (Acari: Phytoseiidae). *Acta Societatis Zoologicae Bohemicae,* 65, pp. 247-251.

Kristiansen E. & Zachariassen K. E. (2005) The mechanism by which fish antifreeze proteins cause thermal hysteresis. *Cryobiology* 51, pp. 262–280.

Kukal, O. (1991). Behavioral and physiological adaptations to cold in a freeze-tolerant arctic insect. In: *Insects at Low Temperature.* R. E. Lee and D. L. Denlinger (eds), Chapman and Hall, N.Y.

Kunte, K., Shea, C., Aardema, M. L., Scriber, J. M., Juenger, T. E., Gilbert, L. E., Kronforst, M. R. (2011) Sex chromosome mosaicism and hybrid speciation among tiger swallowtail butterflies. *PLoS Genetics*, 7, pp. e1002274.

Kunzig, R. (2005) Turning point. *Discover,* 26, pp. 26–28.

Larsen, K. J. & Lee, R. E. (1994) Cold tolerance including rapid cold-hardening and inoculative freezing of fall migrant monarch butterflies in Ohio*. Journal of Insect Physiology*, 40, pp. 859-864.

Layne, J. R., & Kuharsky, D. K. (2000) Triggering of cryoprotectant synthesis in the woolly bear caterpillar (*Pyrrharctica Isabella* Lepidoptera: Arctiidae). *Journal of Experimental Zoology,* 286, pp. 367-371.

Leather, S. R. (1993) Overwintering in 6 Arable Aphid Pests - a Review with Particular Relevance to Pest-Management. *Journal of Applied Entomology-Zeitschrift Fur Angewandte Entomologie,* 116, pp. 217-233.

Lebouvier, M., Laparie, M., Hulle, M., Marais, A., Cozic, Y., Lalouette, L., Vernon, P., Candresse, T., Frenot, Y., Renault, D. (2011) The significance of the sub-Antarctic Kerguelen Islands for the assessment of the vulnerability of native communities to climate change, alien insect invasions and plant viruses. *Biological Invasions*, 13, pp. 1195–1208.

Lee, R.E. & Denlinger, D. L. (1985) Cold tolerance in diapausing and non-diapausing stages of the flesh fly, *Sarchophaga-crassipalpis. Physiological Entomology*. 10, pp. 309-315.

Lee, K. Y. & Denlinger, D. L. (1996) Diapause- regulated proteins in the gut of pharate first instar of the gypsy moth, *Lymantria dispar*, and the effect of KK-42 and neck ligation on expression. *Journal of Insect Physiology*. 42, pp. 423–431.

Lee, R. E., Chen, C. P., Denlinger, D. L. (1987) A rapid cold-hardening process in insects. *Science,* 238, pp. 1415–1417.

Lee, R. E. (1991) Principles of insect low temperature tolerance, in: *Insects at low temperature* (Lee, R. E. & Denlinger, D. L., eds), Chapman & Hall, New York. pp. 17-46.

Lee, R. E. & Denlinger, D. L. (eds.) (1991) *Insects at low temperature*. New York: Chapman and Hall.

Lee, R .E., Lee, M. L., Strong-Gunderson, J. M. (1993) Insect cold hardiness and ice nucleating active organisms including their potential use for biological control. *Journal of Insect Physiology,* 39, pp. 1-12.

Lee, R. E., Costanzo, J. P. (1998) Biological ice nucleation and ice distribution in cold-hardy ectothermic animals. *Annual Review of Physiology*, 60, pp. 55–72.

Lee, R.E., Hankison, S. J. (2003) Acquisition of freezing tolerance in early autumn and seasonal changes in gall water content influence inoculative freezing of gall fly larvae, *Eurosta solidaginis* (Diptera, Tephritidae). *Journal of Insect Physiology*, 49, pp. 385–393.

Levy, O., Dayan, T., Rotics, S., Kronfeld-Schor, N., Arita, H. (2012) Foraging sequence, energy intake and torpor: an individual-based field study of energy balancing in desert golden spiny mice. *Ecology Letters*, 15, pp. 1240–1248.

Li, Y. P. Gong, H., Park, H.Y. (2000) Biochemistry and physiology of overwintering in the mature larva of the pine needle gall midge, *Thecodiplosis japonensis* (Diptera : Cecidomyiidae) in Korea. *Cryoletters*, 21, pp. 149 -156

Li, Y. P., Goto, M., Ito, S., Sato, Y., Sasaki, K., Goto, N. (2001) Physiology of diapause and cold hardiness in the overwintering pupae of the fall webworm *Hyphantria cunea* (Lepidoptera: Arctiidae) in Japan. *Journal of Insect Physiology,* 47, pp. 1181-1187.

Li, Y. P., Goto, M., Ding, L., Tsumuki, H. (2002) Diapause development and acclimation regulating enzymes associated with glycerol synthesis in the Shonai ecotype of the rice stem borer larva, *Chilo suppressalis* walker. *Journal of Insect Physiology,* 48, pp. 303–310.

Li, A., Denlinger, D. L. (2008) Rapid cold hardening elicits changes in brain protein profiles of the flesh fly, *Sarcophaga crassipalpis*. *Insect Molecular Biology*, 17, pp. 565–572.

Lindblad, M., & Areno, P. (2002) Temporal and spatial population dynamics of *Psammotettix alienus*, a vector of wheat dwarf virus. International *Journal of Pest Management*, 48, pp. 233–238.

Lindblad, M., & Sigvald, R. (2004). Temporal spread of wheat dwarf virus and mature plant resistance in winter wheat. *Crop Protection*. 23, pp. 229–234.

Lindgren, E., and R. Gustafson. (2001) Tick-borne encephalitis in Sweden and climate change. *Lancet.* 358, pp. 16–18.

Liu, Z., Gong, P., Wu, K., Sun, J., Li, D. (2006) A true summer diapause induced by high temperatures in the cotton bollworm, *Helicoverpa armigera* (Lepidoptera: Noctuidae). *Journal of Insect Physiology*. 52, pp. 1012–1020.

Liu, Q., Cai, J., Chang, Y., Gu, Y., Guo, Y., Wang, X., Zhong, M., Wang, X., Yang, L., Wu, K., Lan, L., Wang, J., Chen, Y. (2011) Identification of forensically important blow fly species (Diptera: Calliphoridae) in China by mitochondrial cytochrome oxidase I gene differentiation. *Insect Science*, 18, pp. 554–564.

Lole, M. J. (2005) Nuisance flies and landfill activities: an investigation at a West Midlands landfill site. *Waste Management & Research*, 23, pp. 420–428.

Lopez, C., Eizaguirre, M., Albajes, R. (1995) Diapause detection and monitoring in the Mediterranean corn stalk borer. *Physiological Entomology.* 20, pp. 330–336.

Lundheim, R. & Zachariassen, K. E. (1993) Water balance strategies of over-wintering beetles in relation to strategies for cold tolerance, *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology,* 163, pp. 1-4.

Ludwig, C., & Viant, M. R. (2010). Two-dimensional J-resolved NMR spectroscopy: review of a key methodology in the metabolomics toolbox. *Phytochemical Analysis: PCA*, 21, pp. 22–32.

Macario, A. J., Lange, M., Ahring, B. K.,DeMacario, E.C., (1999) Stress genes and proteins in the archaea. *Microbiology and Molecular Biology Reviews*, 63, 923–967.

MacArthur, R. H. (1972) *Geographical ecology : patterns in the distribution of species.* Harper & Row, New York.

MacDonald, G. M. (2010) Global warming and the Arctic: a new world beyond the reach of the Grinnellian niche? *The Journal of Experimental Biology*, 213, pp. 855–861.

MacLeod, J. & Donnelly, J. (1957) Some ecological relationships of natural populations of *calliphorine* blowflies*. Journal of Animal Ecology*, 26, 135–170.

MacMillan, H. A., Walsh, J. P., Sinclair, B. J. (2009) The effects of selection for cold tolerance on cross-tolerance to other environmental stressors in *Drosophila melanogaster*. *Insect Science*, 16, pp. 263–276.

MacRae, T. H. (2002) Gene expression, metabolic regulation and stress tolerance during diapause. *Cellular and Molecular Life Sciences,* 67, pp. 2405-2424.

Maes, S., Machtelinckx, T., Moens, M., Gregoire, J.C., & Clercq, P. (2012) The influence of acclimation, endosymbionts and diet on the supercooling capacity of the predatory bug *Macrolophus pygmaeus. BioControl*, 57, pp. 643–651.

Mansingh, A. & Smallman, B. N. (1971) Influence of Temperature on Photoperiodic Regulation of Diapause in *Saturniids*. *Journal of Insect Physiology,* 17, pp. 1735-1742.

Marshall, K. E., Sinclair, B. J. (2011) The sub-lethal effects of repeated freezing in the woolly bear caterpillar *Pyrrharctia isabella*. *The Journal of Experimental Biology*. 214, pp. 1205–1212.

Marshall, K. E., Sinclair, B. J. (2012a) The impacts of repeated cold exposure on insects. *The Journal of Experimental Biology*, 215, pp. 1607–1613.

Marshall, K.E., Sinclair, B.J. (2012) Threshold temperatures mediate the impact of reduced snow cover on overwintering freeze-tolerant caterpillars. *Die Naturwissenschaften*, 99, pp. 33–41.

Masaki, S. (1980) Summer diapause. *Annual Review of Entomology*. 25, pp 1-25

Matthews, P.G.D. & White, C.R. (2011) Discontinuous gas exchange in insects: is it all in their heads? *American Naturalist*, 177, 130–134.

Matuszewski, S., Bajerlein, D., Konwerski, S., Szpila, K. (2008) An initial study of insect succession and carrion decomposition in various forest habitats of Central Europe. *Forensic Science International*, 180, pp. 61–69.

Matuszewski, S., Bajerlein, D., Konwerski, S., Szpila, K. (2010) Insect succession and carrion decomposition in selected forests of Central Europe. Part 2: Composition and residency patterns of carrion fauna. *Forensic science international*, 195, pp. 42–51.

McWatters, H. G. & Saunders, D. S. (1996) The influence of each parent and geographic origin on larval diapause in the blow fly, *Calliphora vicina*. *Journal of Insect Physiology,* 42, pp. 721-726.

McWatters, H. G. & Saunders, D. S. (1998) Maternal temperature has different effects on the photoperiodic response and duration of larval diapause in blow fly (*Calliphora vicina*) strains collected at two latitudes. *Physiological Entomology,* 23, pp. 369-375.

Meier, P. & Zettel, J. (1997) Cold hardiness in *Entomobrya nivalis* (Collembola, Entomobryidae): Annual cycle of polyols and antifreeze proteins, and antifreeze triggering by temperature and photoperiod. *Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology*, 167, pp. 297–304.

Menu, F. (1993) Diapause Development in the Chestnut Weevil *Curculio-Elephas*. *Entomologia Experimentalis Et Applicata,* 69, pp. 91-96.

Menu, F. & Debouzie, D. (1993) Coin-Flipping Plasticity and Prolonged Diapause in Insects - Example of the Chestnut Weevil *Curculio-Elephas* (Coleoptera, Curculionidae). *Oecologia,* 93, pp. 367-373.

Meng, F. G., Park, Y. D., Zhou, H. M. (2001) Role of proline, glycerol, and heparin as protein folding aids during refolding of rabbit muscle creatine kinase. *International Journal of Biochemistry & Cell Biology,* 33, pp. 701-709.

Meola, R. W. & Adkisson, P. L. (1977) Release of prothoracicotropic hormone and potentiation of developmental ability during diapause in the bollworm, *Heliothis zea*. *Journal of Insect Physiology*. 23, pp. 683–688.

Michaud, M. R. & Denlinger, D.L. (2006) Oleic acid is elevated in cell membranes during rapid cold hardening and pupal diapause in the flesh fly, *Sarcophaga crassipalpis*. *Journal of Insect Physiology*. 52, pp. 1073–1082.

Michaud, M. R. & Denlinger, D. L. (2007) Shifts in the carbohydrate, polyol, and amino acid pools during rapid cold-hardening and diapause-associated cold-hardening in flesh flies (*Sarcophaga crassipalpis*): a metabolomic comparison. *Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology*, 177, pp. 753-763.

Michaud, M. R., Benoit, J. B., Lopez-Martinez, G., Elnitsky, M. A., Lee, R. E., Denlinger, D. L. (2008) Metabolomics reveals unique and shared metabolic changes in response to heat shock, freezing and desiccation in the Antarctic midge, *Belgica antarctica. Journal of Insect Physiology*, 54, pp. 645–655.

Michaud, M. R. & Denlinger, D. L. (2010) *Low temperature biology of insects.* Cambridge: Cambridge University Press.

Miles, J. E., Bale, J. S., Hodkinson, I. D. (1998) Life cycle regulation in the heather psyllid *Strophingia ericae*: responses to temperature and photoperiod. *Physiological Entomology*, 23, pp. 376–381.

Miller, L. K. (1978) Freezing tolerance in relation to cooling rate in an adult insect. *Cryobiology* 15, pp. 345–349

Milonas, P. G. & Savopoulou-Soultani, M. (2000) Diapause induction and termination in the parasitoid *Colpoclypeus florus* (Hymenoptera: Eulophidae): Role of photoperiod and temperature. *Annals of the Entomological Society of America*, 93, pp. 512-518.

Mitchell, K. A., Hoffmann, A. A. (2010) Thermal ramping rate influences evolutionary potential and species differences for upper thermal limits in Drosophila. *Functional Ecology* 24, pp. 694–700.

Mizrahi, T., Heller, J., Goldenberg, S., Arad, Z. (2010) Heat shock proteins and resistance to desiccation in congeneric land snails. *Cell stress & chaperones, 15,* pp. 351–363.

Monari, M., Foschi, J., Rosmini, R., Marin, M. G., Serrazanetti, G. P. (2011) Heat shock protein 70 response to physical and chemical stress in *Chamelea gallina*. *Journal of Experimental Marine Biology and Ecology*, 397, pp. 71–78.

Morey, A. C., Hutchinson, W. D., Venette, R. C., Burkness, E. C. (2012) Cold hardiness of *Helicoverpa zea* (Lepidoptera: Noctuidae) pupae. *Environmental entomology*. 41, pp. 172-179.

Morgan, T. D. & Chippendale, G. M. (1983) Free Amino-Acids of the Hemolymph of the Southwestern Corn-Borer and the European Corn-Borer in Relation to Their Diapause. *Journal of Insect Physiology*, 29, pp. 735-740.

Mugnano, J. A., Lee, R. E., Taylor, R. T. (1996) Fat body cells and calcium phosphate sperules induce ice nucleation in the freeze tolerant larvae of the gall fly *Eurosta solidaginis* (Diptera, Tephritidae). *Journal of Experimental Biology,* 199, pp. 465-471.

Musolin, D. L. (2007) Insects in a warmer world: ecological, physiological and life-history responses of true bugs (Heteroptera) to climate change. *Global Change Biology*, 13, pp. 1565–1585.

Musolin, D. L., Tougou, D., Fujisaki, K. (2010). Too hot to handle? Phenological and life-history responses to simulated climate change of the southern green stink bug *Nezara viridula* (Heteroptera: Pentatomidae). Global Change Biology, 16, pp. 73–87.

Nath, B. S., Suresh, A., Varma, B. M., Kumar, R. P. (1997) Changes in protein metabolism in hemolymph and fat body of the silkworm, *Bombyx mori* (Lepidoptera: Bombycidae) in response to organophosphorus insecticides toxicity. *Ecotoxicology and Environmental Safety*, 36, pp. 169–173.

Natural History Museum. (2011). *Available from*: http://www.nhm.ac.uk/nature-online/species-of-the-day/scientific-advances/forensics/calliphora-vicina/taxonomy/index.html [accessed 05/06/2011].

Nedved, O. (1993) Comparison of cold hardiness in two ladybird beetles (Coleoptera: Coccinellidae) with contrasting hibernation behaviour. *European Journal of Entomology*, 90, pp. 465-470.

Neven, L. (1999) Cold hardiness adaptations of codling moth, *Cydia pomonella*. *Cryobiology*, 38, pp. 43–50.

Nibouche, S. (1998) High temperature induced diapause in the cotton bollworm Helicoverpa armigera. *Entomologia Experimentalis Et Applicata,* 87, pp. 271-274.

Niederegger, S., Pastuschek, J., Mall, G. (2010) Preliminary studies of the influence of fluctuating temperatures on the development of various forensically relevant flies. *Forensic Science International*, 199, pp. 72–78.

Nissim, I., Hardy, M., Pleasure, J., States, B. (1992) A mechanism of glycine and alanine cytoprotective action: stimulation of stress-induced HSP70 mRNA. *Kidney international*, 42, pp. 775–782.

Nuorteva, P. & Laurikainen, E. (1964) Synanthropy of blowflies (Diptera: Calliphoridae) on the island of Gotland, Sweden. *Annales Entomologici Fennici,* 30, pp. 187-190.

Nuorteva, P. (1965) The flying activity of blowflies (Dipt., Calliphoridae) in subarctic conditions. *Annales Entomologici Fennici.* 31, pp. 242-245.

Novotny, V., Basset, Y., Miller, S. E., Weiblen, G. D., Bremer, B., Cizek, L. and Drozd, P. (2002) Low beta diversity of herbivorous insects in tropical forests. *Nature*, 416, pp. 841-844.

O'Neill, S. L., Giordano, R., Colbert, A. M., Karr, T. L., Robertson, H. M. (1992) 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. *Proceedings of the National Academy of Sciences of the United States of America,* 89, pp. 2699–2702.

Overgaard, J., Sorensen, J. G., Petersen, S. O., Loeschcke, V., Holmstrup, M. (2005) Changes in membrane lipid composition following rapid cold hardening in *Drosophila melanogaster*. *Journal of Insect Physiology,* 51, pp. 1173–1182.

Overgaard, J., Malmendal, A., Sorensen, J. G., Bundy, J. G., Loeschcke, V., Nielsen, N. C., Holmstrup, M. (2007) Metabolomic profiling of rapid cold hardening and cold shock in *Drosophila melanogaster*. *Journal of Insect Physiology*, 53, pp. 1218–1232.

Parmesan, C., Ryrholm, N., Stefanescu, C., Hillk, J.K., Thomas, C. D., Descimon, H., Huntleyk, B., Kaila, L., Kullberg, J., Tammaru, T., Tennent, W. J., Thomas, J. A., Warren, M. (1999) Poleward shifts in geographical ranges of butterfly species associated with regional warming. *Nature*, 399, pp. 579–583.

Parsons, P. A. (1970) Genetic heterogeneity in natural populations of *Drosophila melanogaster* for ability to withstand desiccation. *Theoretical and Applied Genetics,* 40, pp. 261-266.

Parsons, H. M., Ludwig, C., Gunther, U. L., Viant, M. R. (2007) Improved classification accuracy in 1- and 2-dimensional NMR metabolomics data using the variance stabilising generalised logarithm transformation. *BMC Bioinformatics*, 8, 234.

Payne, J. A. (1965) A summer carrion study of the baby pig *Sus scrofa* Linnaeus. *Ecology*, 46, pp. 592–602.

Pedersen, K. S., Kristensen, T. N., Loeschcke, V., Petersen, B. O., Duus, J. O., Nielsen, N. C., Malmendal, A. (2008) Metabolomic signatures of inbreeding at benign and stressful temperatures in *Drosophila melanogaster*. *Genetics*, 180, pp. 1233–1243.

Petersen, C. R., Holmstrup, M., Malmendal, A., Bayley, M., Overgaard, J. (2008) Slow desiccation improves dehydration tolerance and accumulation of compatible osmolytes in earthworm cocoons (*Dendrobaena* octaedra Savigny). *The Journal of Experimental Biology*, 211, pp. 1903–1910.

Perez-Banon, C., Juan, A., Petanidou, T., Marcos-Garcia, M. A., Crespo, M. B. (2003) The reproductive ecology of *Medicago citrina* (Font Quer) Greuter (Leguminosae): a bee-pollinated plant in Mediterranean islands where bees are absent. *Plant Systematics and Evolution*, 241, pp. 29–46.

Pfister, T. D. & Storey, K. B. (2002) Purification and characterization of protein phosphatase-1 from two cold-hardy goldenrod gall insects. *Archives of Insect Biochemistry and Physiology,* 49, pp. 56-64.

Philip, B. N., Yi, S. X., Elnitsky, M. A., Lee, R. E. (2008) Aquaporins play a role in desiccation and freeze tolerance in larvae of the goldenrod gall fly, *Eurosta solidaginis. Journal of Experimental Biology*, 211, pp. 1114–1119.

Pike, N. & Kingcombe, R. (2009). Antibiotic treatment leads to the elimination of *Wolbachia* endosymbionts and sterility in the diplodiploid collembolan *Folsomia candida*. *BMC Biology*, *7*, pp. 54.

Pohjoismaki, J. L. O., Karhunen, P. J., Goebeler, S., Saukko, P., Saaksjarvi, I. E. (2010) Indoors forensic entomology: colonization of human remains in closed environments by specific species of sarcosaprophagous flies. *Forensic Science International*, 199, pp. 38–42.

Powell, J.A. (2001). Longest insect dormancy: Yucca moth larvae (Lepidoptera: Prodoxidae) metamorphose after 20, 25, and 30 years in diapause. *Annals of the Entomological Society of America,* 94, 677–680; 49, pp. 56-64.

Powell, S. J., Bale, J. S. (2004) Cold shock injury and ecological costs of rapid cold hardening in the grain aphid *Sitobion avenae* (Hemiptera: Aphididae). *Journal of Insect Physiology*, 50, pp. 277–284.

Poynton, H. C., Taylor, N. S., Hicks, J., Colson, K., Chan, S. R., Clark, C., Scanlan, L., Loguinov, A. V., Vulpe, C., Viant, M. R. (2011) Metabolomics of Microliter Hemolymph Samples Enables an Improved Understanding of the Combined Metabolic and Transcriptional Responses of *Daphnia magna* to Cadmium. *Environmental Science & Technology,* 45, pp. 3710-3717.

Predel, R., Nachman, R. J. (2001) Efficacy of native FXPRLamides (pyrokinins) and synthetic analogs on visceral muscles of the American cockroach. *Journal of Insect Physiology,* 47, pp. 287–293.

Pruitt, N. L., Lu, C. (2012) Seasonal changes in phospholipid class and class-specific fatty acid composition associated with the onset of freeze tolerance in third-instar larvae of *Eurosta solidaginis*. *Physiological and Biochemical Zoology: PBZ*, 81, pp. 226–34.

Pruthi, H. S. (1932) Colonisation of the sea by insects*. Nature*, 130, pp. 312-312.

Pujol-Luz, J. R. & Barros-Cordeiro, K. B. (2012) Intra-puparial development of the females of *Chrysomya albiceps* (Wiedemann) (Diptera, Calliphoridae). *Revista Bresileira de Entomologica,* 56, pp. 269-272.

Pullin, A. S., Bale, J. S. (1989) Influence of diapause and temperature on cryoprotectant synthesis and cold hardiness in pupae of *Pieris brassicae*. *Comparative Biochemistry and Physiology* , 94A, pp. 499-503.

Pullin, A. S. (1996) Physiological relationships between insect diapause and cold tolerance: Coevolution or coincidence? *European Journal of Entomology,* 93, pp. 121-129.

Qin, W., Neal, S. J., Robertson, R. M., Westwood, J. T., Walker, V. K. (2005) Cold hardening and transcriptional change in *Drosophila melanogaster*. *Insect Molecular Biology*, 14, pp. 607–613.

Quinn, P. J. (1985) A Lipid-Phase Separation Model of Low-Temperature Damage to Biological-Membranes. *Cryobiology,* 22, pp. 128-146.

Rader, R., Howlett, B. G., Cunningham, S. A., Westcott, D. A., Newstrom-Lloyd, L. E., Walker, M. K., Teulon, D. A. J., Edwards, W. (2009) Alternative pollinator taxa are equally efficient but not as effective as the honeybee in a mass flowering crop. *Journal of Applied Ecology*, 46, pp. 1080–1087.

Ragland, G. J., Fuller, J., Feder, J. L., Hahn, D. A. (2009) Biphasic metabolic rate trajectory of pupal diapause termination and post-diapause development in a tephritid fly. *Journal of Insect Physiology.* 55, pp. 344-350.

Ramadan, Z., Jacobs, D., Grigorov, M., Kochhar, S. (2006) Metabolic profiling using principal component analysis, discriminant partial least squares, and genetic algorithms. *Talanta*, 68, pp. 1683–91.

Ramløv, H. & Westh, P. (1992). Survival of the cryptobiotic eutardigrade *Adorybiotus coronifer* during cooling to 196^{o}C effect of cooling rate, trehalose level, and short-term acclimation. *Cryobiology*. 29, pp. 125-130.

Ramløv, H. & Westh, P. (1993) Ice formation in the freeze tolerant alpine weta *Hemideina maori* Hutton (Orthoptera: Stenopelmatidae). *Cryo-Letters*, 14, pp. 169-276.

Ramløv, H. & Lee, R. E. (2000) Extreme resistance to desiccation in overwintering larvae of the gall fly, *Eurosta solidaginis* (Diperta, Tephritidae). *Journal of Experimental Biology*, 789, pp. 783–789.

Randolph, S. E. (2000) Ticks and tick-borne disease systems in space and from space. *Advances in Parasitology,* 47, pp. 217–243.

Randolph, S. E. (2009) Perspectives on climate change impacts on infectious disease. *Ecology,* 90, pp. 927-931.

Reibe, S., Schmitz, J., Madea, B. (2009) Molecular identification of forensically important blowfly species (Diptera: Calliphoridae) from Germany. *Parasitology research,* 106, pp. 257–61.

Reibe, S. & Madea, B. (2010) How promptly do blowflies colonise fresh carcasses? A study comparing indoor with outdoor locations. *Forensic Science International*, 195, pp. 52–57.

Renault, D., Salin, C., Vannier, G., Vernon, P. (2002) Survival at low temperatures in insects: what is the ecological significance of the supercooling point? *CryoLetters*, 23, pp. 217-228.

Renkema, J. M., Lynch, D. H., Cutler, G. C., MacKenzie, K., Walde, S. D. (2012) Emergence of Blueberry Maggot Flies (Diptera: Tephritidae) from Mulches and Soil at Various Depths. *Environmental Entomology*. 41, pp. 370-376.

Reznik, S. Y., Voinovich, N. D., & Vaghina, N. P. (2011). Maternal regulation of *Trichogramma embryophagum* Htg. (Hymenoptera: Trichogrammatidae) diapause: Photoperiodic sensitivity of adult females. *Biological Control*, 57, 158–162.

Richard, D. S. & Saunders, D. S. (1987) Prothoracic Gland-Function in Diapause and Nondiapause *Sarcophaga argyrostoma* and *Calliphora vicina*. *Journal of Insect Physiology,* 33, pp. 385-392.

Rinehart, J. P. & Denlinger, D. L. (2000) Heat-shock protein 90 is down-regulated during pupal diapause in the flesh fly, *Sarcophaga crassipalpis*, but remains responsive to thermal stress. *Insect Molecular Biology,* 9, pp. 641-645.

Rinehart, J. P., Yocum, G. D., Denlinger, D. L. (2000). Thermotolerance and rapid cold-hardening ameliorate the negative effects of brief exposures to high or low temperatures on fecundity in the flesh fly, *Sarcophaga crassipalpis. Physiological Entomology*, 25, pp. 330–336.

Rinehart, J. P., Li, A., Yocum, G. D., Robich, R. M., Hayward, S. A. L., Denlinger, D. L. (2007) Up-regulation of heat shock proteins is essential for cold survival during insect diapause *Proceedings of the National Academy of Sciences of the United States of America*, 104, pp. 11130-11137.

Ring, R. A. (1968) Termination of diapause in larva of *Lucilia caesar* L. (Diptera, Calliphridae). *Canadian Journal of Entomology*, 46, pp. 335-344.

Ring, R. (1982). Freezing-tolerant insects with low supercooling points. *Comparative Biochemistry and Physiology*, 73a, pp. 605-612.

Ring, R. A., Danks, H. V. (1994) Desiccation and cryoprotection: overlapping adaptations. *CryoLetters* 15, pp. 181–190.

Robinson, R. A. & Sutherland, W.J. (2002) Post-war changes in arable farming and biodiversity in Great Britain. *Journal of Applied Ecology*, 39, pp. 157–176.

Roques, A. (1989) New results and some thinking about significance and induction of prolonged diapause in cone insects with particular references to the larch cone fly (Lasiomma *melania*) and to the Douglas-Fir seed chalcid (*Megastigmus spermotrop*hus). *In: Proceeding cone and seed insects working party conference, Working Party S2.07-01, IUFRO, 26-30 June 1988* (ed G.E. Miller), pp. 64-81, Victoria BC, Canada.

Roux, O., Gers, C., Legal, L. (2006) When, during ontogeny, waxes in the blowfly (*Calliphoridae*) cuticle can act as phylogenetic markers. *Biochemical Systematics and Ecology*, 34, pp. 406–416.

Roversi, P. F., Cosi, E., Irdani, T. (2008) Chill sensitivity and cryopreservation of eggs of the greater wax moth *Galleria mellonella* (Lepidoptera: Pyralidae). *Cryobiology*, 56, pp. 1–7.

Roy, D. B. & Sparks, T. H. (2000) Phenology of British butterflies and climate change. *Global Change Biology*, 6, pp. 407–416.

Rudolph, A. S. & Crowe, J. H. (1985) Membrane Stabilization During Freezing - the Role of 2 Natural Cryoprotectants, Trehalose and Proline. *Cryobiology,* 22, pp. 367-377.

Rybczynski, R., Snyder, C. A, Hartmann, J., Gilbert, L. I., Sakurai, S. (2009) *Manduca sexta* prothoracicotropic hormone: evidence for a role beyond steroidogenesis. *Archives of Insect Biochemistry and Physiology*, 70, pp. 217–29.

Sabanoglu, B. & Sert, O. (2010) Determination of *Calliphoridae* (Diptera) fauna and seasonal distribution on carrion in Ankara province. *Journal of Forensic Sciences*, 55, pp. 1003–1007.

Salt, R. W. (1957). Natural occurrence of glycerol in insects and its relation to their ability to survive freezing. *Canadian Entomologist*, 89, pp. 491.

Salt, R. W. (1961) Principles of Insect Cold-Hardiness. *Annual Review of Entomology,* 6, pp. 55-60.

Salt, R. W. (1966) Factors influencing nucleation in supercooled insects. *Canadian Journal of Zoology,* 44, pp. 117–133.

Saravanakumar, R., Ponnuvel, K. M., Qadri, S. M. H. (2008) Expression of metabolic enzyme genes and heat-shock protein genes during embryonic development in diapause and non-diapause egg of multivoltine silkworm *Bombyx mori*. *Biologia*, 63 , pp. 737–744.

Saunders, D. S. (1987) Maternal Influence on the Incidence and Duration of Larval Diapause in *Calliphora vicina*. *Physiological Entomology,* 12, pp. 331-338.

Saunders, D. S. (1997) Under-sized larvae from short-day adults of the blow fly, *Calliphora vicina*, side-step the diapause programme. *Physiological Entomology.* 22, pp. 249–255.

Saunders, D. S. & Hayward, S. A. L. (1998) Geographical and diapause-related cold tolerance in the blow fly, *Calliphora vicina*. *Journal of Insect Physiology,* 44, pp. 541-551.

Saunders, D. S. & Cymborowski, B. (2003) Selection for high diapause incidence in blow flies (*Calliphora vicina*) maintained under long days increases the maternal critical daylength: some consequences for the photoperiodic clock. *Journal of Insect Physiology.* 49, pp. 777-784.

Sawyer, A. J., Tauber, M. J., Tauber, C. A., Ruberson, J. R. (1993) Gypsy moth (Lepidoptera: Lymantriidae) egg development: a simulation analysis of laboratory and field data. *Ecological Modelling.* 66, pp. 121-155.

Schimpf, N. G., Matthews, P. G. D., Wilson, R. S., White, C. R. (2009) Cockroaches breathe discontinuously to reduce respiratory water loss. *The Journal of Experimental Biology*, 212, pp. 2773–2780.

Schroeder, H., Klotzbach, H., Elias, S., Augustin, C., Pueschel, K. (2003) Use of PCR–RFLP for differentiation of *calliphorid* larvae (Diptera, Calliphoridae) on human corpses. *Forensic Science International*, 132, pp. 76–81.

Schwarz, J. J. & Gries, G. (2010) 2-Phenylethanol: context-specific aggregation or sex-attractant pheromone of *Boisea rubrolineata* (heteroptera: Rhopalidae). *Canadian Entomologist*, 142, pp. 489-500.

Scott, P. A. & Kettleborough, J. A. (2002) Origins and estimates of uncertainty in predictions of twenty-first century temperature rise. *Nature*, 416, pp. 723-726.

Serkova, N. J., Rose, J. C., Epperson, L. E., Carey, H. V., Martin, S. L. (2007) Quantitative analysis of liver metabolites in three stages of the circannual hibernation cycle in 13-lined ground squirrels by NMR. *Physiological Genomics*, 31, pp. 15-24.

Sgolastra, F., Bosch, J., Molowny-Horas, R., Maini, S., Kemp, W. P. (2010) Effect of temperature regime on diapause intensity in an adult-wintering *Hymenopteran* with obligate diapause. *Journal of Insect Physiology,* 56, pp. 185-194.

Shen, Y., Gu, J., Huang, L. H., Zheng, S. C., Liu, L., Xu, W. H., Feng, Q. L., Kang, L. (2011) Cloning and expression analysis of six small heat shock protein genes in the common cutworm, *Spodoptera litura*. *Journal of Insect Physiology*, 57, pp. 908–914.

Shimizu, T., & Kawasaki, K. (2001) Geographic variability in diapause response of Japanese Orius species. *Entomologia Experimentalis et Applicata*, 98, pp. 303–316.

Shimizu, T., Kanamori, Y., Furuki, T., Kikawada, T., Okuda, T., Taka- hashi, T., Mihara, H., Sakurai, M. (2010) Desiccation-induced struc- turalization and glass formation of group 3 late embryogenesis abundant protein model peptides. *Biochemistry*, 49, 1093–1104.

Shintani, Y. & Ishikawa, Y. (2007) Relationship between rapid cold-hardening and cold acclimation in the eggs of the yellow-spotted longicorn beetle, *Psacothea hilaris*. *Journal of Insect Physiology*, 53, pp. 1055–1062.

Shintani, Y. (2009) Artificial selection for responsiveness to photoperiodic change alters the response to stationary photoperiods in maternal induction of egg diapause in the rice leaf bug *Trigonotylus caelestialium*. *Journal of Insect Physiology*, 55, 818–824.

Siegert, K.J., (1986) The effects of chilling and integumentary injury on carbohydrate and lipid metabolism in diapause and non-diapause pupae of *Manduca sexta*. *Comparative Biochemistry and Physiology.* 85A, pp. 257–262.

Sinclair, B.J. (1999). Insect cold tolerance; How many kinds of frozen? *European Journal of Entomology*, 96, pp. 157-164.

Sinclair, B. J., Worland, M. R., Wharton, D. A. (1999) Ice nucleation and freezing tolerance in New Zealand alpine and lowland weta, *Heminoptera spp*. (Orthoptera, Stenopelmatidae). *Physiological Entomology*, 24, pp. 56-63.

Sinclair, B. J. & Sjursen, H. (2001) Cold tolerance of the Antarctic springtail *Gomphiocephalus hodgsoni* (Collembola, Hypogastruridae). *Antarctic Science,* 13, pp. 271-279.

Sinclair, B. J., Addo-Bediako, A, Chown, S. L. (2003a) Climatic variability and the evolution of insect freeze tolerance. *Biological Reviews of the Cambridge Philosophical Society*, 78, pp. 181–195.

Sinclair, B. J., Jaco-Klok, C., Scott, M. B., Terblanche, J. S., Chown, S. L. (2003d) Diurnal variation in supercooling points of three species of *Collembola* from Cape Hallett, Antarctica. *Journal of Insect Physiology*, 49, pp. 1049–1061.

Sinclair, B .J. & Chown, S .L. (2005) Climatic variability and hemispheric differences in insect cold tolerance: support from southern Africa. *Functional Ecology*. 19, pp. 214–221.

Sinclair, B .J., Gibbs, A. G., Roberts, S. P. (2007) Gene transcription during exposure to, and recovery from, cold and desiccation stress in *Drosophila melanogaster*. *Insect Molecular Biology,* 16, pp. 435–443.

Sjursen, H., Bayley, M., Holmstrup, M. (2001) Enhanced drought tolerance of a soil-dwelling springtail by pre-acclimation to a mild drought stress. *Journal of Insect Physiology*, 47, pp. 1021–1027.

Slachta, M., Vambera, J., Zahradnickova, H., Kostal, V. (2002) Entering diapause is a prerequisite for successful cold- acclimation in adult *Graphosoma lineatum* (Heteroptera: Pentatomidae). *Journal of Insect Physiology*, 48, pp. 1031-1039.

Slama, K. (2012) A new look at the comparative physiology of insect and human hearts. *Journal of Insect Physiology*, 58, pp. 1072–1081.

Slone, D. H., Gruner, S. V. (2007) Thermoregulation in larval aggregations of carrion-feeding blow flies (Diptera: Calliphoridae). *Journal of Medical Entomology*, 44, pp. 516–523.

Somme, L. (1996) The effect of prolonged exposures at low temperatures in insects. *CryoLetters*, 17, pp. 341-346.

Sorensen, J. G., Kristensen, T. N., Loeschcke, V. (2003) The evolutionary and ecological role of heat shock proteins. *Ecology Letters*, 6, pp. 1025–1037.

Soudi, S., & Moharramipour, S. (2012) Seasonal patterns of the thermal response in relation to sugar and polyol accumulation in overwintering adults of elm leaf beetle, *Xanthogaleruca luteola* (Coleoptera: Chrysomelidae). *Journal of Thermal Biology*, 37, 384–391.

Soula, B. & Menu, F. (2003) Variability in diapause duration in the chestnut weevil: mixed ESS, genetic polymorphism or bet-hedging? *Oikos,* 100, pp. 574-580.

Stanic B., Jovanovic-Galovic, A., Blagojevic, D. P., Grubor-Lajsic, G., Worland, R., Spasic, M. B. (2004) Cold hardiness in *Ostrinia nubilalis* (Lepidoptera: Pyralidae): glycerol content, hexose mono- phosphate shunt activity, and antioxidative defense system. *European Journal of Entomology*, 101, pp. 459–466.

Stevens, J. & Wall, R. (1996) Species, sub-species and hybrid populations of the Blowflies *Lucilia cuprinia* and *Lucilia sericata* (Diptera: Calliphoridae). *Proceeding: Biological Sciences*, 263, pp. 1335–1341.

Stevens, J. & Wall, R. (2001) Genetic relationships between blowflies (*Calliphoridae*) of forensic importance. *Forensic Science International,* 120, pp. 116-123.

Stevens, J. R., Wall, R., Wells, J. D. (2002) Paraphyly in Hawaiian hybrid blowfly populations and the evolutionary history of anthropophilic species. *Insect Molecular Biology*, 11, pp. 141–148.

Stoeckli, S., Hirschi, M., Spirig, C., Calanca, P., Rotach, M. W., Samietz, J. (2012) Impact of Climate Change on Voltinism and Prospective Diapause Induction of a Global Pest Insect - *Cydia pomonella* (L.). *PloS one*, 7, pp. e35723.

Storey, K. B. & Storey, J. M. (1988) Freeze tolerance in animals. *Physiological Reviews*, 68, pp. 27–84.

Storey, K. B. & Storey, J. M. (1996) Natural freezing survival in animals. *Annual Review of Ecological Systems,* 27, pp. 365–386.

Storey, K. B. (2007) Anoxia tolerance in turtles: metabolic regulation and gene expression. *Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology*, 147, pp. 263–276.

Strong-Gunderson, J. M., Lee, R. E., Lee M. R., Riga, T. J. (1990) Ingestion of ice-nucleating bacteria increases the supercooling point of the lady beetle *Hippodamia convergens*. *Journal of Insect Physiology*, 36, pp. 153–157.

Su, Y. L., Lu, Z. Z., Song, J. & Miao, W. (2007) Effect of overwintering aggregation on energy metabolism in the firebug, *Pyrrhocoris apterus* (Heteroptera: Pyrrhocoridae). *Acta Entomologica Sinica,* 50, pp. 1300-1303.

Sushikumar, J. & Ray, P. (2010) Activity enhancement of *Zygogramma bicolorata,* a biocontrol agent of *Parthenium hysterophorus,* by temperature regulated diapause aversion. *Biocontrol Science and Technology*, 20, pp. 903-908.

Tachibana, S. I. & Numata, H. (2004) Parental and direct effects of photoperiod and temperature on the induction of larval diapause in the blow fly *Lucilia sericata*. *Physiological Entomology*, 29, pp. 39-44.

Tachibana, S. I., Numata, H., Goto, S. G. (2005) Gene expression of heat-shock proteins (Hsp23, Hsp70 and Hsp90) during and after larval diapause in the blow fly *Lucilia sericata*. *Journal of Insect Physiology,* 51, pp. 641-647.

Takeda, K., Musolin, D. L., Fujisaki, K. (2010) Dissecting insect responses to climate warming: overwintering and post-diapause performance in the southern green stink bug, *Nezara viridula*, under simulated climate-change conditions, *Physiological Entomology*, 35, pp. 343–353.

Tanaka, K. (1997) Evolutionary relationship between diapause and cold hardiness in the house spider, *Achaearanea tepidariorum* (Araneae: Theridiidae). *Journal of Insect Physiology,* 43, pp. 271–274.

Tanaka, S. (2000) The role of moisture in the control of diapause, mating and aggregation in a tropical insect. *Entomological Science* 3, pp. 147–155.

Tauber, M. J., Tauber, C. A., 1973. Quantitative response to daylength during diapause in insects. *Nature*, 244, pp. 296–297.

Tauber, M. J. & Tauber, C. A. (1976) Insect seasonality: diapause maintenance, termination, and postdiapause development. *Annual Reviews of Entomology,* 21, pp. 81–107.

Tauber, M. J., Tauber, C. A., Masaki, S. O. (1986) *Seasonal adaptations of insects,* New York, Oxford University Press.

Teets, N .M., Peyton, J. T., Ragland, G. J., Colinet, H., Renault, D., Hahn, D. A, Denlinger, D. L. (2012) Combined transcriptomic and metabolomic approach uncovers molecular mechanisms of cold tolerance in a temperate flesh fly. *Physiological genomics*, 44, pp. 764–77.

Terblanche, J. S., Clusella-Trullas, S., Chown, S. L. (2010) Phenotypic plasticity of gas exchange pattern and water loss in *Scarabaeus spretus* (Coleoptera: Scarabaeidae): deconstructing the basis for metabolic rate variation. *Journal of Experimental Biology*. 213, pp. 2940–2949.

Terlecky, S. R., Chiang, H. L., Olson, T. S., Dice, J. F. (1992) Protein and peptide binding and stimulation of invitro lysosomal proteolysis by the 73-Kda heat-shock cognate protein. *Journal of Biological Chemistry,* 267, pp. 9202-9209.

Thomson, J. D. (2010) Flowering phenology, fruiting success and progressive deterioration of pollination in an early-flowering geophyte. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, 365, pp. 3187–99.

Thorne, M. A. S., Worland, M. R., Feret, R., Deery, M. J., Lilley, K. S., Clark, M. S. (2011) Proteomics of cryoprotective dehydration in *Megaphorura arctica* Tullberg 1876 (Onychiuridae: Collembola). *Insect Molecular Biology*, 20, pp. 303–10.

Tiziani, S., Lodi, A., Ludwig, C., Parsons, H. M., Viant, M. R. (2008) Effects of the application of different window functions and projection methods on processing of 1H J-resolved nuclear magnetic resonance spectra for metabolomics. *Analytica Chimica Acta*, 610, pp. 80–88.

Tomcala, A., Tollarová, M., Overgaard, J., Simek, P., Kostál, V. (2006) Seasonal acquisition of chill tolerance and restructuring of membrane glycerophospholipids in an overwintering insect: triggering by low temperature, desiccation and diapause progression. *The Journal of Experimental Biology*, 209, pp. 4102–14.

Topp, W. (1990) Selection for optimal monovoltine life cycle in an unpredictable environment. Studies on the beetle *Catops nigricans* Spence (Col., Catopidae). *Oecologia,* 84, pp. 134–141.

Touchette, B. W. & Burkholder, J. M. (2000) Overview of the physiological ecology of carbon metabolism in seagrasses. *Jou*rnal *of Experimental Marine Biology and Ecology*, 250, pp. 169–205.

Tsuji, H. (1958) Studies on the diapause of the Indian meal moth, *Plodia interpunctella* Hubner. I. The influence of temperature on the diapause and the type of diapause. *Japanese Journal of Applied Entomology and Zoology*, 2, pp. 17–23.

Tsumuki, H., Konno, H., Maeda, T., Okamoto, Y. (1992) An ice nucleating active fungus isolated from the gut of the rice stem borer, *Chilo suppressalis* Walker (Lepidoptera: Pyralidae). *Journal of Insect Physiology*, 38, pp. 119-125.

Tsvetkova, N. M., Horvath, I., Torok, Z., Wolkers, W. F., Balogi, Z., Shigapova, N., Crowe, L. M., Tablin, F., Vierling, E., Crowe, J. H. Vigh, L. (2002) Small heat-shock proteins regulate membrane lipid polymorphism. *Proceedings of the National Academy of Sciences of the United States of America,* 99, pp. 13504-13509.

Turba, M. E., Zambon, E., Zannoni, A., Russo, S., Gentilini, F. (2012) Detection of Wolbachia DNA in blood for diagnosing filaria-associated syndromes in cats. *Journal of Clinical Microbiology,* 50, pp. 2624–30.

Tursman, D., Duman, J. G., Knight, C. A. (1994) Freeze tolerance adaptations in the centipede, *Lithobius forficatus. Journal of Experimental Zoology*, 268, pp. 347-353.

Tuzun, A., Dabiri, F., Yuksel, S. (2010) Preliminary study and Identification of insects' species of forensic importance in Urmia, Iran. *African Journal of Biotechnology*, 9, pp. 3649–3658.

Uehara, H., Senoh, Y., Yoneda, K., Kato, Y., Shiomi, K. (2011) An FXPRLamide neuropeptide induces seasonal reproductive polyphenism underlying a life- history tradeoff in the tussock moth. *PLoS One,* 6: e24213.

Varkonyi, G., Hanski, I., Rost, M., Itamies, J. (2002) Host-parasitoid dynamics in periodic boreal moths. *Oikos,* 98, pp. 421-430.

van Asch, M., van Tienderen, P. H., Holleman, L. J. M., Visser, M. E. (2007) Predicting adaptation of phenology in response to climate change, an insect herbivore example. *Global Change Biology,* 13, pp. 1596–1604.

Vaz Nunes, M. & Saunders, D. S. (1989) The Effect of Larval Temperature and Photoperiod on the Incidence of Larval Diapause in the Blowfly *Calliphora vicina*. *Physiological Entomology,* 14, pp. 471-474.

Veerman, A. (1992) Diapause in Phytoseiid Mites - a Review. *Experimental & Applied Acarology,* 14, pp. 1-60.

Venable, D. L. & Lawlor, L. (1980) Delayed germination and dispersal in desert annuals - escape in space and time. *Oecologia*, 46, pp. 272-282.

Vernon, P. & Vannier, G. (2002) Evolution of freezing susceptibility and freezing tolerance in terrestrial arthropods. *Comptes Rendus Biologies,* 325, pp. 1185-1190.

Vesala, L. & Hoikkala, A. (2011) Effects of photoperiodically induced reproductive diapause and cold hardening on the cold tolerance *of Drosophila montana*. *Journal of insect physiology,* 57, pp. 46–51.

Viant, M.R. (2008) Environmental Metabolomics Using ^{1}H-NMR Spectroscopy. In:. Martin, C. C. (Eds.) *Environmental Genomics,* Humana Press

Vinogradova, E. B. & Zinovjev, K. B. (1972) Maternal Induction of Larval Diapause in Blowfly, *Calliphora-Vicina*. *Journal of Insect Physiology,* 18, pp. 2401-2404.

Vinogradova, E. B. (1974) The pattern of reactivation of diapause larvae in the blowfly, *Calliphora vicina*. *Journal of Insect Physiology,* 20, pp. 2487-2496

Vinogradova, E. B. (1975) Intraspecific variability in the reactions controlling larval diapause in *Calliphora vicina* R-D (Diptera: Calliphoridae). *Entomological Review*, 54, pp. 11–20

Vinogradova, E. B. & Marchenko,M. I. (1984) The use of temperature parameters of fly growth in the medicolegal practice. *Sudebno-Meditsinkskaya Ekspertiza*, 27, pp. 16–19.

Vinogradova E. B. (1986) *Geographical variation and ecological control of diapause in flies. The Evolution of Insect Life-Cycles* (ed. by F Taylor & R Karban), pp. 35–47. Springer, New York, NY, USA.

Vinogradova, E. (2009) Methods of short-term storage of cultures of the blowfly *Calliphora Vicina*: R.-D. (Diptera, Calliphoridae). *Entomological Review,* 89, pp. 1019-1024.

Vinod, K. V. & Sabu, T. K. (2010) Dormancy-inducing factors of rubber litter beetle, *Luprops tristis* (Coleoptera: Tenebrionidae). *Insect Science,* 17, pp. 47-51.

Voss, K., Combs, B., Patterson, K.R., Binder, L.I., Gamblin, T.C. (2012) Hsp70 alters tau function and aggregation in an isoform specific manner. *Biochemistry*, 51, pp. 888–98.

Wageninger. (2011). Available from: http://www.infojardin.com/foro/showthread.php?t=198762&page=156 [Accessed 05/09/2011]

Wall, R., French, N. P., Morgan, K. L. (1992) Effects of temperature on the development and abundance of the sheep blowfly *Lucilia sericata* (Diptera: Calliphoridae). *Bulletin of Entomological Research,* 82, pp. 125–131.

Wan, C., Kiessling, V., Tamm, L. K. (2008) Coupling of cholesterol-rich lipid phases in asymmetric bilayers. *Biochemistry*, 47, pp. 2190–8.

Wang, G., Li, S., Lin, H., Brumbaugh, E. E., Huang, C. (1999) Effects of various numbers and positions of cis double bonds in the sn-2 acyl chain of phosphatidylethanolamine on the chain-melting temperature. *The Journal of Biological Chemistry*, 274, pp. 12289–99.

Wang, H. S. & Kang, L., (2005) Effect of cooling rates on the cold hardiness and cryoprotectant profiles of locust eggs. *Cryobiology*, 51, pp. 220–229.

Watanabe, M., & Tanaka, K. (1998) Adult diapause and cold hardiness in *Aulacophora nigripennis* (Coleoptera: Chrysomelidae). *Journal of Insect Physiology*, 44, pp. 1103–1110.

Waterhouse, D. F. & Paramonov, S. J. (1950) The status of the two species of *Lucilia* (Diptera: Calliphoridae) attacking sheep in Australia. *Australian Journal of Science Research*, 3, pp. 310–336.

Watson, M. J. O. & Hoffmann, A. A. (1996) Cross-Generation Effects for Cold Resistance in Tropical Populations of *Drosophila melanogaster* and *Drosophila simulans*. *Australian Journal of Zoology,* 43, pp. 51 – 58.

Werren, J. H., Baldo, L., Clark, M. E. (2008). *Wolbachia*: master manipulators of invertebrate biology. *Nature Reviews*, 6, pp. 741-751.

Werren, J. H. (1997). Biology of *Wolbachia*. *Annual Review of Entomology*, 42, pp. 587-609.

Wharton, D. A., Pow, B., Kristensen, M., Ramløv, H., Marshall, C. J. (2009) Ice-active proteins and cryoprotectants from the New Zealand alpine cockroach, *Celatoblatta quinquemaculata. Journal of Insect Physiology*, 55, pp. 27–31.

Whitworth, T. L., Dawson, R. D., Magalon, H., Baudry, E. (2007) DNA barcoding cannot reliably identify species of the blowfly genus Protocalliphora (Diptera: Calliphoridae). *Proceedings. Biological sciences / The Royal Society*, 274, pp. 1731–9.

Wigglesworth, V. B. (1972). *The Principles of Insect Physiology.* 7th Edition. Chapman and Hall.

Williams, C. M. (1956) Physiology of insect diapause an endocrine mechanism for the influence of temperature on the diapausing pupa of the Cecropia silkworm. *Biological Bulletin,* 110, pp. 201-218.

Williams, I. H. (1994) The dependence of crop production within the European Union on pollination by honey bees. *Agricultural Zoology Reviews,* 6, pp. 229–257.

Williams, K. A. & Villet, M. H. (2006) A new and earlier record of Chrysomya megacephala in South Africa , with notes on another exotic species, *Calliphora vicina* (Diptera: Calliphoridae). *African Invertebrates*, 47, pp. 347–350.

Wilson, E. O. (1992) *The diversity of life,* Cambridge, Mass., Harvard University Press.

Wilson, P., Heneghan, A., Haymet, A. D. (2003) Ice nucleation in nature: supercooling point (SCP) measurements nd the role of heterogeneous nucleation. *Cryobiology*, 46, pp. 88–98.

Wolda, H. (1988) Insect seasonality. Why? *Annual Review of Ecology and Systematics,* 19, pp. 1–18.

Wolschin, F. & Gadau, J. (2009) Deciphering proteomic signatures of early diapause in Nasonia. *PloS one*, 4, e. 6394.

Wood, F. E. & Nordin, J. H. (1980) Activation of the Hexose-Monophosphate Shunt During Cold-Induced Glycerol Accumulation by Protophormia terranovae. Insect Biochemistry, 10, pp. 87-93.

Woods, H.A. & Smith, J.N. (2010) Universal model for water costs of gas exchange by animals and plants. *PNAS*, 107, 8469–8474.

Woodman, J. D. (2012) Cold tolerance of the Australian spur-throated locust*, Austracris guttulosa*. *Journal of insect physiology*, 58, pp. 384–90.

Woods, H. A. & Smith, J. N. (2010) Universal model for water costs of gas exchange by animals and plants. *Proceedings of the National Academy of Sciences of the United States of America*, 107, pp. 8469–8474.

Wooldridge, J., Scrase, L., Wall, R. (2007) Flight activity of the blowflies, *Calliphora vomitoria* and *Lucilia sericata*, in the dark. *Forensic Science International*, 172, pp. 94–7.

Worland, M. R., Grubor-Lajsic, G., Montiel, P. O. (1998) Partial desiccation induced by sub-zero temperatures as a component of the survival strategy of the Arctic collembolan *Onychiurus arcticus* (Tullberg). *Journal of Insect Physiology,* 44, pp. 211-219.

Worland, M. R. (2005) Factors that influence freezing in the sub-Antarctic springtail *Tullbergia antarctica*. *Journal of Insect Physiology*, 51, pp. 881–94.

Worley, B., Halouska, S., Powers, R. (2013) Utilities for quantifying separation in PCA/PLS-DA scores plots. *Analytical Biochemistry*, 433, pp. 102–4.

Wright, A. J., Fellows, G. A., Griffiths, J. R., Wilson, M., Bell, B. A., Howe, F. A. (2010) Ex-vivo HRMAS of adult brain tumours: metabolite quantification and assignment of tumour biomarkers. *Molecular Cancer*, 9, e. 66.

Wu, H., Southam, A. D., Hines, A., Viant, M. R. (2008) High-throughput tissue extraction protocol for NMR- and MS-based metabolomics. *Analytical Biochemistry, 372,* 204–212.

Wu, S. B., Wang, M. Q., Zhang, G. (2010) Effects of putrescine on diapause induction and intensity, and post-diapause development of *Helicoverpa armigera*. *Entomologia Experimentalis et Applicata*, 136, pp. 199–205.

Xu, W. H., Lu, Y.X., Denlinger, D. L. (2012) Cross-talk between the fat body and brain regulates insect developmental arrest. *Proceedings of the National Academy of Sciences of the United States of America*, 109, pp. 14687–14692.

Yamashita O. & Hasegawa K. 1985. Embryonic diapause, pp. 407–434. In Kerkut G.A. & Gilbert L.I. (eds), Comprehensive Insect Physiology, Biochemistry and Pharmacology, Pergamon Press, Oxford.

Yan-Le, S. & Zhao-Zhi, L. (2007) Effect of overwintering aggregation on energy metabolism in the firebug*, Pyrrhocoris apterus* (Heteroptera : Pyrrhocoridae). *Acta Entomologica Sinica.* 50, pp. 1300-1303.

Ye, Y., Wang, X., Zhang, L., Lu, Z., Yan, X. (2012) Unraveling the concentration-dependent metabolic response of Pseudomonas sp. HF-1 to nicotine stress by ^{1}H NMR-based metabolomics. *Ecotoxicology*, 21, pp. 1314–24.

Yi, S. X., Benoit, J. B., Elnitsky, M. A., Kaufmann, N., Brodsky, J. L., Zeidel, M. L., Denlinger, D. L., Lee, R. E. (2011) Function and immuno-localization of aquaporins in the Antarctic midge *Belgica antarctica*. *Journal of Insect Physiology*, 57, pp. 1096–105.

Yocum, G. D., Joplin, K. H. & Denlinger, D. L. (1998) Upregulation of a 23 kDa small heat shock protein transcript during pupal diapause in the flesh fly*, Sarcophaga crassipalpis*. *Insect Biochemistry and Molecular Biology,* 28, pp. 677-682.

Yoder, J. A. & Denlinger, D. L. (1991) Water balance in flesh fly pupae and water vapor absorption associated with diapause. *Journal of Experimental Biology*, 157, pp. 273–286.

Yoder, J. A, Benoit, J. B., Denlinger, D. L., Rivers, D. B. (2006) Stress-induced accumulation of glycerol in the flesh fly, *Sarcophaga bullata*: evidence indicating anti-desiccant and cryoprotectant functions of this polyol and a role for the brain in coordinating the response. *Journal of Insect Physiology*, 52, pp. 202–14.

Yoder, J. A, Benoit, J. B., LaCagnin, M. J., Hobbs, H. H. (2011) Increased cave dwelling reduces the ability of cave crickets to resist dehydration. *Journal of Comparative Physiology. B, Biochemical, Systemic, and Environmental Physiology*, 181, pp. 595–601.

Yoshimi, T., Odagiri, K., Hiroshige, Y., Yokobori, S., Takahashi, Y., Sugaya, Y., Miura, T. (2009) Induction profile of HSP70-cognate genes by environmental pollutants in Chironomidae. *Environmental Toxicology and Pharmacology*, 28, pp. 294–301.

Young, S. R., & Block, W. (1980) Experimental studies on the cold tolerance of *Alaskozetes antarcticus*. *Journal of Insect Physiology*. 26, pp. 227-234.

Young, J. C., Moarefi, I., Hartl, F. U. (2001) Hsp90: a specialized but essential protein-folding tool. *Journal of Cell Biology,* 154, pp. 267-273.

Zachariassen, K. E. & Hammel, H. T. (1976) Nucleating agents in the haemolymph of insects tolerant to freezing. *Nature.* 262, pp. 285-287.

Zachariassen, K. E. & Husby, J. A. (1982) Antifreeze effect of thermal hysteresis agents protects highly supercooled insects. *Nature,* 298, pp. 865-867.

Zachariassen, K. E. (1985) Physiology of cold tolerance in insects. *Physiological Reviews*, 65, pp. 799–832.

Zachariassen, K. E. & Maloiy, G. M. O. (1989) Water balance in beetles as an indicator of environmental humidity. *Fauna Narvegica*, 36B, pp. 37-31.

Zachariassen, K. E., Kristiansen, E. (2000) Ice nucleation and antinucleation in nature. *Cryobiology*, 41, pp. 257–79.

Zhang, Q., Lu, Y. X., Xu, W. H. (2012) Integrated proteomic and metabolomic analysis of larval brain associated with diapause induction and preparation in the cotton bollworm, *Helicoverpa armigera*. *Journal of Proteome Research*. 11, pp. 1042–53.

Zheng, G., Li, P., Pemberton, R., Luo, Y. (2011) Mixed bumblebee and blowfly pollination of *Cypripedium flavum* (Orchidaceae) in Sichuan, China. *Ecological Research*, 26, pp. 453–459.

Zhu, K., Ding, X., Julotok, M., Wilkinson, B. J. (2005) Exogenous isoleucine and fatty acid shortening ensure the high content of anteiso-C 15: 0 fatty acid required for low-temperature growth of *Listeria monocytogenes*. *Applied and Environmental Microbiology*, 71, pp. 8002–8007.

Zhu, K., Woodall, C. W., Clark, J. S. (2012) Failure to migrate: lack of tree range expansion in response to climate change. *Global Change Biology*, 18, pp. 1042–1041